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<b>(54) Title:</b> <i>VIBRIO CHOLERA</i> E STRAINS DEFECTIVE IN <i>irgA</i> EXPRESSION, AND CHOLERA VACCINES DERIVED THEREFROM  <b>(57) Abstract</b>  A <i>Vibrio cholerae</i> cell harboring a mutation which inhibits or prevents expression in the cell of a functional <i>irgA</i> gene product; a purified preparation of such mutant cells; and a cholera vaccine incorporating such mutant cells.		

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- 1 -

**VIBRIO CHOLERAЕ STRAINS DEFECTIVE IN *irgA*  
EXPRESSION, AND CHOLERA VACCINES DERIVED THEREFROM**

Background of the Invention

The field of the invention is *Vibrio cholerae* mutations. This invention was made with governmental support (Public Health Service grant AI27329 awarded by the National Institute of Allergy and Infectious Diseases), and the government has certain rights to the invention.

Despite more than a century of research on cholera, this disease remains a major health problem in developing countries of Asia and Africa. It causes over 30,000 deaths per year in Bangladesh alone, and perhaps over 200,000 worldwide (Holmgren et al., 1989). One approach to the development of an effective cholera vaccine is the generation of *Vibrio cholerae* strains which are attenuated yet immunogenic: that is, they lack the virulent characteristics of the wild-type bacterium, while maintaining the latter organism's ability to stimulate an immune response that can protect the host from subsequent infection with wild-type *V. cholerae*. By the use of modern genetic engineering methods, mutant strains of *V. cholerae* have been created in which specific genes (or critical portions of the genes) contributing to the virulence of the wild type organism are deleted. For example, deletion mutations in each of the two alleles encoding the toxic A subunit of cholera toxin (*ctxA*), but not in the genes encoding the immunogenic B subunit, has yielded a number of mutant strains that have been tested in human volunteers [Mekalanos et al., 1983; Mekalanos, U.S. Patent No. 4,882,278 (herein incorporated by reference); Kaper et al., 1984; Levine et al., 1988a,b; Herrington et al., 1988]. In addition, deletion mutations in two other *V.*

- 2 -

*cholerae* genes, *tcpA* and *toxR*, have been described (Taylor et al., 1987).

#### Summary of the Invention

It has been found that a strain of *V. cholerae* with an insertional inactivation of the single *irgA* locus has about a ten-fold reduced ability to colonize the intestines of test animals, compared to the colonization ability of the wild-type parental strain. In addition, this *irgA* mutant is about 100-fold less virulent than the wild-type parent strain, as assessed by LD<sub>50</sub> (the dosage level which is lethal to 50% of the test animals which receive it). When this mutation is combined in a single strain of *V. cholerae* with mutations which inactivate both of the *ctxA* alleles [or any other gene(s) the inactivation of which substantially diminishes virulence while maintaining immunogenicity], the resulting strain would exhibit significantly less virulence in test subjects than would a strain harboring only the *irgA* mutation or only the *ctxA* mutations. Lessened virulence means that the inoculated animal would be less likely to develop the side effects, such as diarrhea and fever, which are frequently seen with other live attenuated cholera vaccines. Mutations in genes other than *irgA*, such as *irgB*, which also result in a decrease in IrgA production, would also be useful in producing a strain of *V. cholerae* with substantially lowered virulence.

The invention features a *V. cholerae* cell (or a purified preparation of such cells) harboring a mutation which inhibits or prevents expression in the cell of a functional *irgA* gene product. This mutation could be in the *irgA* gene itself, in the *irgB* gene, or in any other gene which affects production of functional IrgA protein. For maximal stability of the mutation, it is preferably a deletion of a substantial amount of the affected gene

- 3 -

(e.g., at least 25% of the coding sequence of the affected gene, and more preferably at least 50%). This mutation may be the only such virulence-inhibiting mutation in the cell, or may be combined in the cell with

5 a second mutation which inhibits production in the cell of a second *V. cholerae* virulence factor (besides IrgA), and potentially with additional mutations which have the effect of reducing the levels of still other virulence factors. *V. cholerae* virulence factors which have been

10 identified so far include cholera toxin (subunits A and B), neuraminidase, hemolysin, and certain specific adhesins including toxin coregulated pilus. One such second mutation that is potentially useful in the invention is a *ctxA* deletion mutant, such as in *V.*

15 *cholerae* strain 0395-N1 (Mekalanos, U.S. Patent No. 4,882,278). In this strain, a substantial amount of the coding sequence of each of the two *ctxA* alleles has been deleted, so that no functional cholera toxin A subunit is made by these cells.

20 As used herein, the term "functional *irgA* gene product" is taken to mean an outer membrane protein of approximate molecular weight 77 kDa (as determined by polyacrylamide gel electrophoresis) which functions as a *V. cholerae* virulence factor: that is, strains in which

25 the 77 kDa protein is produced at a decreased level compared to the parental (or a wild-type) strain are less virulent *in vivo* than the parental (or wild-type) strain; such virulence can be conveniently assayed using the *in vivo* mouse model described in the Examples. A mutation

30 is herein said to inhibit expression of a functional *irgA* gene product if a substantially lower amount (e.g., 50% or less) of the 77 kDa *irgA* gene product is produced by the mutant strain under low-iron culture conditions than is produced by the parental strain under identical

35 culture conditions. (*In vitro* assays employing

- 4 -

appropriate low-iron conditions are described in the Examples below.) The mutation is said to prevent expression of a functional *irgA* gene product if no 77 kDa *irgA* gene product is detectable in a polyacrylamide gel electrophoresis analysis of outer membrane proteins produced by the mutant strain under low-iron conditions, as described in Example 1 below. A mutation is said to inhibit the production of a second virulence factor if, as a result of the mutation, the level of the second virulence factor in the mutant cell is decreased in comparison with the level in a cell of the parental strain, as measured by (a) a significant (e.g., at least 50%) decrease in virulence in the mutant strain compared to the parental strain, and (b) a significant (e.g., at least 50%) decrease in the amount of the polypeptide identified as the second virulence factor in the mutant strain compared to the parental strain. A mutation which leaves the cell incapable of producing a detectable amount of the virulence factor of interest (assayed by standard methods such as gel electrophoresis and autoradiography under conditions such as those utilized in the Examples set forth below) is said to prevent such production. A preparation of cells is termed "substantially purified" if contaminating *V. cholerae* cells without the desired mutant genotype constitute less than 1% (and preferably less than 0.1%) of the total number of cells in the preparation.

Also within the invention is a cholera vaccine made up of the mutant cells of the invention, and a method for inducing immunity to cholera in a mammal by administering to the mammal an effective amount of such cholera vaccine. The cells used for the vaccine are preferably alive and thus capable of colonizing the intestines of the inoculated animal, and may be combined

- 5 -

if desired with vaccines targeted at other illnesses to make a single, multi-valent vaccine.

Other features and advantages of the invention will be apparent from the following detailed description,  
5 and from the claims.

### Detailed Description

The drawings are first described.

#### Drawings

Fig. 1 is an autoradiogram of an electrophoresis  
10 gel of outer membrane proteins for *V. cholera* 0395 wild-type cells grown in high-iron medium (lane 1); 0395 wild-type cells grown in low-iron medium (lane 2); MBG40 cells grown in high-iron medium (lane 3); and MBG40 cells grown in low-iron medium (lane 4).

15 Fig. 2 is a Southern blot analysis of chromosomal DNA from 10 mutant strains, digested with *Eco* RV and probed with a  $^{32}\text{P}$ -labeled internal fragment of *TnphoA*, where lane 1 is from mutant strain MBG18; lane 2 is from MBG19; lane 3 is from MBG20; lane 4 is from MBG21; lane 5  
20 is from MBG24; lane 6 is from MBG25; lane 7 is from MBG25; lane 8 is from MBG34; lane 9 is from MBG37; and lane 10 is from MBG40; the numbers to the left of the gel indicate the sizes (in kilobases) of standards used.

Fig. 3 is an illustration of *in vitro* growth  
25 curves of 0395 wild-type cells and MBG40 cells (A) in LB medium with and without added dipyridyl, and (B) in T medium with and without added iron; where closed circles represent 0395 wild-type in high-iron medium, open circles represent 0395 wild-type in low-iron medium,  
30 closed triangles represent MBG40 in high-iron medium, and open triangles represent MBG40 in low-iron medium.

Fig. 4 is a diagram illustrating the construction of plasmids utilized in Example 2, where: the lightly-stippled bar represents MBG40 chromosomal DNA contained

- 6 -

in the insert; the open bar represents DNA from *TnphoA*; the darkly-stippled bar (Km) represents the kanamycin resistance gene carried on *TnphoA*; the cross-hatched bar (Ap) represents the ampicillin resistance gene carried on pBR322; the arrow indicates the direction of transcription of *irgA*; and B=BamHI, Bg=BglII, N=NheI, and Nr=NruI.

Fig. 5 is a Western blot analysis of proteins from MBG40 and 0395 prepared after growth in low- and high-iron media and probed with anti-PhoA antibody, where lane 1 represents whole-cell proteins of 0395 grown in high iron; lane 2, whole-cell proteins of MBG40 grown in high iron; lane 3, periplasmic extract of MBG40 grown in low iron; and lane 4, whole-cell proteins of MBG40 grown in low iron; the sizes of molecular weight standards (in kDa) are indicated on the left.

Fig. 6 is a restriction map of *irgA* and upstream chromosomal DNA in pMBG59.

Fig. 7 is a Northern blot analysis of RNA from MBG40 and 0395, probed with a <sup>32</sup>P-labeled fragment of *irgA*, where lane 1a is 0395 grown in high iron; lane 1b is 0395 grown in low iron; lane 2a is MBG40 grown in high iron; and lane 2b is MBG40 grown in low iron; the positions of single-stranded RNA molecular weight markers (in kb) are indicated on the left.

Fig. 8 is an illustration of the nucleotide sequence of *irgA* upstream of its fusion with *phoA*, the promoter region of *irgA*, and the large region of upstream DNA required for its transcription, starting at the ClaI site, with the first five nucleotides of the *phoA* sequence after the fusion joint with *irgA* indicated by lower case letters at the end of the sequence (corresponds to SEQ ID NO.: 1).

Fig. 9 is an illustration of nucleotide homology between the proposed iron regulatory region of *irgA* and



- 7 -

the Fur box consensus sequence of *E. coli* [5,6], where arrows refer to areas of interrupted dyad symmetry.

Fig. 10 is a hydropathicity plot of the 151-residue amino-terminus of IrgA, where hydrophobicity is indicated by positive (+) data points and hydrophilicity by negative (-) data points, with each data point representing the average of three consecutive residues.

Fig. 11 is an illustration of one region of homology between the peptide sequences of IrgA and FepA, with the number of residues from the amino terminus of each precursor protein to the start of each sequence indicated on the left.

Fig. 12 is a Northern blot analysis of RNA from 0395 and MBG40, probed with an oligonucleotide complementary to the 5' terminus of *irgB*, where lane 1 represents 0395 grown in high iron; lane 2, 0395 grown in low iron; lane 3, MBG40 grown in high iron; and lane 4, MBG40 grown in low iron; the positions of single-stranded RNA molecular weight markers (in kilobases) are indicated on the left.

Fig. 13 is an illustration of the nucleotide sequence of the chromosomal DNA in pMBG59 (reading 5' to 3' from right to left in Fig. 1), starting downstream of the start site of *irgA* transcription and extending up to the ClaI restriction site in pMBG59, with the deduced amino acid sequence of IrgB shown in three-letter code (corresponds to SEQ ID NO.: 2).

Fig. 14 is a depiction of the overlapping, divergent promoters of *irgA* and *irgB*, with the promoter of *irgB* (-35, -10), start site of transcription of *irgB* (\*), Shine-Dalgarno sequence (SD) of *irgB*, and open reading frame of *irgB* indicated on the upper strand, while the corresponding features of *irgA* are noted on the bottom strand.

- 8 -

Fig. 15 is an illustration of the homology between the amino terminus of IrgB and several members of the LysR family of positive transcriptional activators, where amino acids are identified in single letter code and identical residues are enclosed in boxes; the conserved helix-turn-helix domain of these proteins is indicated above the sequences.

### The Mutation

Included within the invention is any *V. cholerae* cell harboring a mutation which inhibits or prevents the cell from expressing a functional *irgA* gene product. This includes all types of inactivating mutations of *irgA* or of any other locus necessary for expression of *irgA*. Such mutations may constitute, for example, insertions, deletions, or replacements of one or a few nucleotides, mutations that could result in frame shifts or in relatively minor changes in critical portions of the affected polypeptide, which in the case of the *irgA* gene is the 77-kDa major iron-regulated outer membrane protein. It is expected that nearly all mutations that result in a change in the amino acid sequence of this protein would decrease the protein's ability to carry out its function, and thus would be within the invention. More preferably, however, the mutation would constitute a more substantial alteration in the affected gene which would be less likely to revert spontaneously to the wild-type phenotype. One such preferred mutation involves an insertion of a relatively long (e.g., 500 or more base pairs) segment of DNA directly into the *irgA* coding sequence, such as is described below in Example 1. Even more stable would be a mutation in which a substantial amount (e.g., more than 25%, and preferably at least 50%) or all of the coding sequence were deleted. If such a mutated gene expressed any polypeptide at all, it would

- 9 -

be nonfunctional and probably degraded by cellular proteases shortly after synthesis. Such a deletion mutation can be accomplished by any of several methods known to those of ordinary skill in the art of genetic manipulations. One such method would begin with an inactivating insertion mutant prepared as described in Example 1, and would then apply the *in vivo* marker exchange technique described in Mekalanos, U.S. Patent No. 4,882,278, to produce the desired deletion mutation.

10           Mutations of a second *V. cholerae* gene, *irgB*, have also been shown to inhibit expression of *irgA*, and thus would be within the invention. The wild-type IrgB protein serves as a positive regulator of *irgA* transcription, so that an inactivating mutation of *irgB* (such as the insertional mutation of *irgB* described in Example 3 below, or a deletion of any portion of *irgB* as described in Example 1) results in a nearly complete elimination of detectable expression of *irgA*. The marker exchange technique discussed above could be used to produce a deletion mutation in an *irgB* gene into which had been introduced an inactivating insertion in accordance with Example 3.

#### Use

25           The *V. cholerae* cells of the invention are useful as sources of immunological protection against cholera, in particular as the basis of a live-cell vaccine capable of colonizing the inoculated animal's intestine and provoking a strong immune reaction. Appropriate dosages and conditions of administration of such a live, attenuated vaccine are as described in Holem et al., Acute Enteric Infections in Children, New Prospects for Treatment and Prevention (1981) Elsevier/North-Holland Biomedical Press, Ch. 26, pp. 443 et seq. (Levine et al.).

- 10 -

Experimental data

Examples 1-3 below describe experimental results  
in support of the claims.

- 11 -

Example 1: Identification of *irgA* gene in *V. cholerae*, and construction of insertion mutation of *irgA* with decreased virulence in vivo

MATERIALS AND METHODS

Bacterial strains. *V. cholerae* 0395 Sm<sup>r</sup> was a gift of John J. Mekalanos, and *E. coli* CC118 has been described previously (6). Mutant strain MBG40 was derived from 0395 by the methods described below. Strains were maintained at -70°C in LB medium (19) containing 15% glycerol.

Media. Two types of media were used to assess the effect of iron concentration on gene expression: (i) LB medium with or without the addition of the iron chelator 2,2-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 0.2 mM and (ii) Tris-buffered medium (T medium) (30) prepared with highly purified water (Barnstead Nanopure water purification system [Sybron, Boston, Mass.]) and supplemented with 4 g of sucrose per liter, with or without the addition of 36 µM FeSO<sub>4</sub>. The concentrations of iron in growth medium were verified by using 1,10-phenanthroline (Aldrich Chemical Co., Inc., Milwaukee, Wis.) (18). By this assay, T medium contained less than 0.5 µM iron and LB medium contained 10 µM iron.

LB agar was used for high-iron plates. Chelex-LB agar supplemented with 0.3% glucose was used for low-iron plates. Chelex-LB agar was prepared by stirring the iron-chelating resin Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) in fivefold concentrated LB medium overnight at 4°C, filtering through Whatman filter paper (no. 1) to remove the resin, and then adding highly purified water and Noble agar (Disco Laboratories, Detroit, Mich.) before sterilization. Streptomycin (100 µg/ml), kanamycin (45 µg/ml), gentamicin (30 µg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (XP) (Amresco; 40 µg/ml) were added where appropriate. XP is a chromogenic substrate for alkaline phosphatase.

Genetic methods. The transposon vector TnphoA was used

to obtain random insertions into the chromosome of *V. cholerae* 0395 by methods previously described (32, 33). Fusion-containing colonies were screened for the *PhoA*<sup>+</sup> phenotype under low-iron growth conditions by the presence or absence of blue color on Chelex-LB agar containing streptomycin, kanamycin, gentamicin, glucose, and XP. They were subsequently screened for iron regulation of alkaline phosphatase activity by streaking on Chelex-LB agar with the same supplements and by adding a filter paper disk spotted with 10  $\mu$ l of 10 mM  $\text{FeSO}_4$  in the streak. Strains that showed a distinct zone of white color surrounding the disk, with blue color peripherally, contained fusions in genes whose expression was negatively regulated by iron.

Confirmation of single *TnphoA* insertions into the chromosome of 0395 was examined by Southern hybridization of digests of chromosomal DNA by using the restriction enzymes *Xba*I and *Eco*RV, which do not cut within the *TnphoA* insert, and probing with an internal fragment of *TnphoA* that had been radioactively labeled by random primer extension with a commercially available kit (Prime Time [International Biotechnologies, Inc.]).

**Assays.** The enzymatic activity of alkaline phosphatase encoded on *TnphoA* permitted the comparison of fusion gene expression when strains were grown in low- versus high-iron media. Strains were grown overnight in LB medium with or without added dipyrldyl and in T medium with or without added  $\text{FeSO}_4$ . Alkaline phosphatase activity was calculated as described previously (21) from measurement of hydrolysis of *p*-nitrophenyl phosphate (Amresco) by permeabilized cells. Activity was expressed in units per  $A_{600}$  of bacterial cells, with 1 U of activity defined as by O'Callaghan et al. (23).

The amount of cholera toxin produced was assayed by using the GM-1-dependent enzyme-linked immunosorbent assay as previously described by Holmgren (15) with slight modifications. Assays were performed on serial dilutions of supernatant fluids from cells grown to saturation in low- and high-iron media at 30°C, using 96-well GM-1 ganglioside-coated microdilution plates.

**Preparation and analysis of outer membrane proteins.** Enriched outer membrane proteins were prepared by procedures previously described (14) from cells grown to late logarithmic phase in LB medium with and without added dipyrldyl. Outer membrane proteins were separated on sodium dodecyl sulfate-10% polyacrylamide gels and stained with Coomassie blue.

**Assessment of virulence by competition and LD<sub>50</sub> assays.** 0395 wild type and mutant strain MBG40 both demonstrated equal plating efficiencies on LB agar and Chelex-LB agar (data not shown). Competition assays between strains were performed essentially as described by Freter et al. (12) and modified by Taylor et al. (33). In vitro competition was determined by growth at 37°C for 24 h in LB medium with dipyrldyl, from a starting density of  $4 \times 10^5$  CFU/ml. In vivo competition was determined by intraintestinal growth in 6- to 7-day-old suckling CD-1 mice (Charles River Breeding Labs, Inc., Wilmington, Mass.) inoculated orally with  $4 \times 10^5$  CFU. The mice were sacrificed 24 h later. The input ratio was approximately 1.0 in both types of competition experiments; competitive indices were corrected for the input ratio. Viable cell counts and the ratio of the two strains were determined by plating dilutions onto Chelex-LB agar containing streptomycin, glucose, and XP and scoring for the *PhoA*<sup>+</sup> blue colony phenotype of the mutant *TnphoA* fusion strain and the *PhoA*<sup>-</sup> white colony phenotype of the wild

- 13 -

type. The in vivo competitive index was the average of values from five individual mouse experiments.

The 50% lethal dose ( $LD_{50}$ ) assays were performed by oral inoculation of 3- to 5-day-old suckling CD-1 mice with various doses of viable bacteria grown in LB medium at 30°C, pelleted, washed twice in LB medium, and suspended in 0.15 M  $\text{NaHCO}_3$  (pH 8.15). Four or more mice were used per dose of bacteria. Survival was determined at 36 h, and results were analyzed as described previously (26).

## RESULTS

*TnphoA* is a derivative of the transposon *Tn5* that contains a portion of *phoA*, the *E. coli* gene for alkaline phosphatase (20). After random insertion of *TnphoA* into chromosomal DNA, those insertions that yield in-frame fusions between a target gene and *phoA* encode hybrid proteins that have a carboxy-terminal fragment of PhoA fused to an amino-terminal portion of the target protein product. These hybrid proteins display alkaline phosphatase activity only if the target gene encodes a protein expressed at the cell surface (a secreted, transmembrane, or outer membrane protein) and so provides the requisite signals for transport of the carboxy-terminal PhoA fragment into the periplasmic space (20). Because most bacterial virulence determinants are expressed at the cell surface, this technique selects for *TnphoA* insertions into such genes (11, 17, 25, 32, 33).

Isolation and characterization of *TnphoA* fusions in iron-regulated genes of *V. cholerae*. (i) Construction and isolation of *TnphoA* insertion mutant strains. After random insertion of *TnphoA* into the chromosome of *V. cholerae* 0395, colonies were screened for the  $\text{PhoA}^+$  phenotype by blue color on a low-iron agar plate containing XP. Of 300 individual blue colonies examined, we obtained 25 *TnphoA* insertion mutant strains whose blue colony phenotype was repressed around an iron-containing disk.

(ii) Alkaline phosphatase assays. Iron regulation of these *TnphoA* gene fusions was confirmed by measuring alkaline phosphatase activity after growth in LB medium with or without added dipyriddy (Table 1). All strains showed a significant increase in alkaline phosphatase activity in low-iron conditions compared with high-iron conditions, with induction ratios ranging from 6-fold (MBG38) to more than 850-fold (MBG40). The differing alkaline phosphatase activities and induction ratios of the 25 mutant strains suggest that we had isolated *TnphoA* insertions into a number of different genes on the *V. cholerae* chromosome. To confirm further the iron regulation of the *TnphoA* gene fusion in MBG40, alkaline phosphatase activity of both this strain and wild-type 0395 was also determined after growth in T medium with and without added iron: alkaline phosphatase activity of MBG40 increased from 1 U after growth in iron-supplemented T medium to 126 U after growth in T medium without added iron. As expected, wild-type 0395 had low levels of alkaline phosphatase activity in both low- and high-iron growth conditions.

(iii) Outer membrane proteins. Since most bacterial virulence determinants are expressed at the cell surface, we analyzed outer membrane proteins for wild-type 0395 and each of the 25 mutant strains after growth in low- and high-iron media. In wild-type 0395, at least four new proteins of apparent molecular mass from 75 to 81 kDa appeared after growth in low-iron conditions compared with high-iron conditions (Fig. 1). The most prominent of these iron-regulated proteins, with an apparent molecular mass of 77 kDa, was lost in two of the 25 mutant strains: MBG15 (data not shown)

and MBG40 (Fig. 1). Several other mutant strains had lost other individual iron-regulated outer membrane protein bands (data not shown).

(iv) Mapping of *TnphoA* insertions to single chromosomal fragments. Because *Xba*I and *Eco*RV do not cut within the *TnphoA* vector, Southern hybridization of *Xba*I- or *Eco*RV-digested chromosomal DNA, probed with an internal fragment of *TnphoA*, was performed for each of the 25 mutant strains. This analysis verified that only single *TnphoA* chromosomal insertions are present in each of the mutant strains (data not shown). Moreover, single *TnphoA* insertions were present in at least eight distinct *Xba*I chromosomal fragments, ranging in size from 9.0 to 30.0 kilobases of DNA (including the inserted *TnphoA* [7.6 kilobases]); there was no hybridization signal for chromosomal DNA of parent 0395 (data not shown). Figure 2 shows a Southern blot of *Eco*RV-digested chromosomal DNA from 10 of the mutant strains, including MBG40.

We selected strain MBG40 for further study for the following two reasons. (i) The expression of the gene fusion in this strain, as measured by alkaline phosphatase activity, was highly regulated by iron, with an induction ratio of more than 850 after growth in low-iron conditions compared with high-iron medium (Table 1). (ii) The *TnphoA* insertion in strain MBG40 was associated with loss of the major 77-kDa iron-regulated outer membrane protein of *V. cholerae* 0395 (Fig. 1). The iron-regulated gene in strain MBG40 that contains the *TnphoA* insertion was designated *irgA*.

In vitro characterization of iron-regulated (*irgA*) fusion strain MBG40. (i) In vitro growth curves. To determine whether MBG40 was defective for growth in vitro, growth curves of 0395 wild type and MBG40 in LB medium with and without added dipyrindyl and in T medium with and without added iron were determined. The two strains grew similarly in each in vitro growth condition (Fig. 3). The enhancement of growth for both strains after the addition of iron to T medium demonstrates that growth in unsupplemented T medium was indeed limited by iron.

(ii) In vitro competition assays. To determine whether MBG40 was able to compete effectively in vitro with 0395 wild type, in vitro competition of the two strains was performed in LB medium with added dipyrindyl. Low-iron media was chosen because previous data has suggested that the intraintestinal environment has limited iron availability (27). The in vitro competitive index of 0.98 (Table 2) con-



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firming the results of the in vitro growth curves, suggesting the absence of an in vitro growth defect in the mutant strain.

(iii) Cholera toxin assays. To control for any differences in cholera toxin production that might affect the virulence of parental and mutant strains under different growth conditions, cholera toxin activity was assayed from the supernatant fluids of 0395 and MBG40 grown to saturation in T medium with and without added iron and in LB medium with and without added dipyrldyl. Cholera toxin activity of the two strains was similar under the various growth conditions (data not shown).

In vivo characterization of iron-regulated (*irgA*) fusion strain MBG40. (i) LD<sub>50</sub> assays. To determine whether *irgA* has a role in virulence of 0395, LD<sub>50</sub> assays of 0395 wild type and MBG40 were performed. The LD<sub>50</sub> of mutant strain MBG40 was  $3 \times 10^5$  bacteria, compared with  $4 \sim 10^3$  bacteria for parental strain 0395 (Table 2). This increase in LD<sub>50</sub> of 2 orders of magnitude clearly suggests that *irgA* is important for virulence in this animal model. Previous data have shown that 0395 strains carrying either a randomly selected chromosomal *TnphoA* insertion or *TnphoA* insertion in the structural gene for the outer membrane protein OmpV maintain full virulence for suckling mice (33), demonstrating that chromosomal *TnphoA* insertion by itself has no effect on virulence.

(ii) In vivo competition assays. Mutant strain MBG40 was tested for a colonization defect by an in vivo competition assay consisting of coinfection of the mutant with parental strain 0395 in suckling mice. The wild-type strain outcompeted the mutant almost 10-fold (in vivo competitive index, 0.11 [Table 2]), suggesting a probable colonization defect in the mutant strain.

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- 18 -

Example 2: Transcriptional regulation of *irgA*, and construction of deletion mutants of gene (*irgB*) encoding the *irgA* transcriptional regulator protein.

Bacterial strains. The *V. cholerae* wild-type strain used in this study was classical Ogawa strain 0395  $Sm^r$  [22]. *V. cholerae* strain MBG40 is 0395 *irgA::Tnp<sub>hoA</sub>* [10]. *E. coli* strain CC118 is a *phoA* deletion derivative of MC1000 [21], and *E. coli* strain SM10 has been described previously [35]. *E. coli* strain DH5 $\alpha$  was obtained from Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, Maryland).

Media. Two types of liquid media were used to assess the effect of iron concentration on gene expression as previously described [10]: (i) LB medium with or without the addition of the iron chelator 2,2-dipyridyl (final concentration 0.2 mM) and (ii) tris-buffered medium (T medium) with or without the addition of 36  $\mu M$   $FeSO_4$ . For *E. coli* strains, T medium was supplemented with thiamine (10  $\mu g/ml$ ) and the L-amino acids arginine and leucine (40  $\mu g/ml$ ). For citrate utilization assays, sodium citrate was added to T media at a final concentration of 10mM.

Solid media containing a normal concentration of iron were made using LB agar. Low-iron LB plates supplemented with 0.3% glucose were made after overnight treatment with Chelex-100 (Bio-Rad Laboratories, Richmond, CA), as described previously [10]. Ampicillin (100  $\mu g/ml$ ), tetracycline (15  $\mu g/ml$ ), kanamycin (45  $\mu g/mg$ ), streptomycin (100  $\mu g/ml$ ), and 5-bromo-4-chloro-3-indolyl phosphate (XP) (Amresco; 40  $\mu g/ml$ ) were added to the media as appropriate. XP is a chromogenic substrate that allows blue-white colony screening on agar plates for alkaline phosphatase activity.

- 19 -

Construction of plasmids. pSBC34 is a derivative of the broad host range plasmid pRK290 [8] modified to contain a gene fusion between the iron-regulated Shiga-like toxin I A gene (slt-IA) from E. coli and phoA, the E. coli gene for alkaline phosphatase. To construct pSBC34, the 5.5 kilobase-pair (kbp) BamHI fragment of pSC105 ( $\Delta$ 2) [5], encompassing the slt-I promoter, slt-IA fused to phoA, and the downstream kanamycin resistance marker, was purified by gel electroelution. This fragment was ligated into the unique BglII restriction site of pRK290, with selection for tetracycline resistance (encoded by pRK290) and kanamycin resistance. pRK290 and its derivatives are not self-transmissible, but can be mobilized at high frequency if supplied with mobilization functions on RP4 in trans. pSBC34 was therefore transformed into SM10, a derivative of E. coli strain C600 that contains a chromosomally-integrated RP4-2 (Tc::Mu). SM10(pSBC34) was conjugated with V. cholerae 0395, with double selection for tetracycline resistance (encoded by pSBC34) and streptomycin resistance (encoded by 0395).

Strain MBG40 contains a chromosomal gene fusion between irgA and phoA, constructed by TnphoA mutagenesis [10]. This gene fusion is contained within a 10 kbp BamHI restriction fragment, extending from a BamHI site in the chromosome approximately 5 kbp upstream of the fusion joint to the unique BamHI site within TnphoA, approximately 5 kbp downstream of the fusion joint. The BamHI site within TnphoA is located downstream of the end of phoA and the kanamycin resistance marker of the transposon.

This gene fusion was cloned into pUC19 as follows. Chromosomal DNA from MBG40 was digested with BamHI and ligated into the BamHI site on

- 20 -

pUC19. The ligation mix was electroporated into DH5a according to methods described below. Colonies containing the correct clone (pMBG47) were isolated by selecting for ampicillin resistance (carried on pUC19) and kanamycin resistance (carried on TnphoA). pMBG47 was then digested with BamHI, and the 10 kbp fragment separated from the parent plasmid by gel electroelution and ligated into the BamHI site on pBR322, creating plasmid pMBG53 (Fig. 4). Presence of the correct insert in pMBG53 was confirmed by sequencing the junction between pBR322 and the TnphoA end of the fragment. Subsequent subcloning of pMBG53 to make pMBG55 and pMBG59 was performed as shown in Figure 4. pMBG59 contains an insert of approximately 1.8 kbp of chromosomal DNA fused to 2.9 kbp of TnphoA. All plasmids were propagated in E. coli strain CC118, which contains a chromosomal deletion of alkaline phosphatase.

Plasmid subclones pMBG57 and pMBG58 were derived from pMBG55 by deletion of portions of the chromosomal insert from each of two BglII restriction sites within the insert to the BamHI restriction site at the junction of the insert with pBR322. Plasmid subclones pMBG102, pMBG105, pMBG109, and pMBG110 were similarly derived from pMBG59 by deletion of portions of the chromosomal insert from AccI, NcoI, ClaI, and HincII restriction sites respectively within the insert to the NruI site in pBR322. Plasmid subclone pMBG103 was derived from pMBG59 by deletion from the BalI site within the chromosomal insert to the BalI site in pBR322.

Genetic methods. Except as noted, plasmid constructs were transformed into E. coli strains and conjugated into V. cholerae 0395 by mobilization from the E. coli strain SM10 by standard techniques

- 21 -

Electroporation used in the transformation of pMBC47 into CC118 was performed in a Gene Pulser (BioRad) according to the manufacturer's protocol. Electroporation conditions were 2.500 V at 25-uF capacitance, producing a time constant of 4.8 ms.

5        Assays. The enzymatic activity of alkaline phosphatase encoded on InphoA permitted screening of plasmid subclones containing the l72A'-'phoA gene fusion for the presence or absence of fusion gene expression under low-iron growth conditions by streaking E. coli strain CC118 containing subclones onto Chelex-LB agar containing XP. Colonies  
10        having a PhoA<sup>+</sup> phenotype are blue on XP, whereas colonies having a PhoA<sup>-</sup> phenotype are white.

Similarly, the enzymatic activity of alkaline phosphatase encoded on InphoA permitted the comparison of fusion gene expression in liquid media when V. cholerae strain MBC40 or E. coli strain CC118 carrying  
15        plasmid subclones of the gene fusion were grown in low- versus high-iron conditions. Strains were grown overnight in T medium with or without added FeSO<sub>4</sub>. Alkaline phosphatase activity was determined as described previously [23] from measurement of hydrolysis of  
20        p-nitrophenyl phosphate (Amresco) by permeabilized cells. Activity was normalized to the A<sub>600</sub> of the bacterial cells and defined as described by O'Callaghan et al. [27].

DNA and RNA analysis. DNA and RNA analysis, including preparation of DNA and RNA, restriction mapping, and Northern blot analysis, were performed according to standard molecular biological techniques [31].  
25        For Northern blot analysis, an equivalent amount of RNA, as calculated from OD<sub>260</sub>, was loaded into each lane. DNA sequencing was performed using Sequenase DNA Sequencing Kit (United States Biochemical

- 22 -

Corporation, Cleveland, Ohio). Primer extension and primer extension sequencing were performed essentially as described by Miller et al. [24], except that the oligonucleotide primers were hybridized to RNA in 0.4 M NaCl and 40 mM Pipes (pH 6.4), without formamide, at 60°C for two hours. RNasin and AMV reverse transcriptase were obtained from Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, Maryland).

Synthetic oligonucleotides for use both as probes for Northern blot analysis and as primers for DNA sequencing and primer extension were the generous gift of Brian Seed.

Protein analysis. Whole cell and periplasmic proteins were prepared following growth in low- and high-iron media as described previously [16]. Proteins were separated on a sodium dodecyl sulfate-12.5% polyacrylamide gel, transferred to a NitroScreen West membrane (Dupont, Boston, Mass.) using a semidry blotting apparatus (Hoefer Scientific Instruments, San Francisco, Ca.). Immunoreactive proteins were visualized by sequential incubation with polyclonal rabbit anti-PhoA antibody (a generous gift of John J. Mekalanos) and goat anti-rabbit immunoglobulin-conjugated alkaline phosphatase (Sigma Chemical Co., St. Louis, Missouri), followed by staining for alkaline phosphatase activity as described previously [25].

Protein database searches. Database searches and protein alignments were done using IBI-Pustell Sequence Analysis software (International Biotechnologies, Inc., New Haven, Conn.) to search the NBRF Protein Database (Release 19) using the FASTP algorithm for protein homology [19]. The hydropathicity index profile of IrgA was calculated by the technique of Kyte-Doolittle [18] using IBI-Pustell Sequence Analysis software.



- 23 -

## RESULTS

Reciprocity of iron regulation between *V. cholerae* and *E. coli*. In order to determine whether or not the mechanism of iron regulation in *V. cholerae* is similar to the mechanism of iron regulation in *E. coli*, we compared iron regulation of the *V. cholerae* gene *irgA* in an *E. coli* background to iron regulation of the *E. coli* gene *slc-1A* in a *V. cholerae* background, using alkaline phosphatase activities of the respective gene fusions following growth in low- and high-iron media (Table 3). These assays demonstrated reciprocal iron regulation of each gene fusion in the heterologous background, suggesting that *V. cholerae* and *E. coli* share a common mechanism of iron regulation. We attempted to transform the *irgA'*-*phoA* gene fusion on pMBG53 into an *E. coli fur*<sup>0</sup> background, but were unable to establish a stable transformant, perhaps due to toxicity of the overexpressed fusion protein in the *fur*<sup>0</sup> background.

Mapping of *irgA* by analysis of protein products and RNA transcripts.

(i) Western blot analysis of *IrgA'*-*PhoA* fusion protein. In order to determine the size of the *IrgA'*-*PhoA* fusion protein, we performed Western blot analysis of whole cell and periplasmic proteins from MBG40 and 0395 following growth in low- and high-iron media, using an anti-*PhoA* antibody probe (Fig. 5). As expected, strain 0395 produced no immunoreactive protein and production of *IrgA'*-*PhoA* by MBG40 was iron-regulated. The *IrgA'*-*PhoA* fusion protein had an apparent molecular mass of 70 kDa in whole cell extracts (Fig. 5, Lane 4), with several smaller immunoreactive species probably representing fusion

- 24 -

protein breakdown products. The fusion protein was transported to the periplasmic space (Fig. 5, Lane 3), but we were unable to resolve with certainty whether the largest band in periplasmic extracts was the same size as in whole cell extracts or slightly smaller, as would occur with proteolytic processing by signal peptidase. Since the PhoA portion of the fusion protein has a predicted molecular mass of approximately 50 kDa, the upstream *IrgA* portion of the fusion protein would have a predicted molecular mass of approximately 20 kDa. Therefore, plasmid pMBG59, which contains approximately 1.8 kbp of DNA upstream of the fusion joint, should contain the 5' terminus of *irgA*, plus more than 1.2 kbp of additional upstream DNA.

(ii) Verification that pMBG59 contains those sequences necessary for the expression and iron regulation of *irgA*. In order to verify that pMBG59 contained the elements essential to expression and iron regulation of *irgA*, we determined alkaline phosphatase activities in low- and high-iron media of *V. cholerae* strain MBG40 and *E. coli* strain CC118 containing plasmid subclones pMBG47, pMBG53, pMBG55, and pMBG59. Alkaline phosphatase activity of MBG40 increased from 1 U after growth in iron-supplemented T media to 126 U after growth in T media without added iron, while activity of CC118 containing each of the plasmid subclones increased from approximately 70 U after growth in iron-supplemented T media to approximately 200 U after growth in T media without iron. Therefore, expression and iron regulation of the gene fusion on each of these plasmid subclones is similar, suggesting that they each contain the DNA sequences necessary for the expression and iron regulation of *irgA*. The difference in induction ratio of iron regulation between CC118 containing the plasmid subclones and MBG40 is

- 25 -

probably due to a plasmid copy number effect and less efficient regulation of a V. cholerae gene in an E. coli background.

(iii) Deletion subcloning and mapping of fusion gene expression and iron regulation. In order to determine the extent of chromosomal

5 DNA upstream of the fusion joint required for fusion gene expression and iron regulation, alkaline phosphatase assays were performed on CC118 containing plasmid deletion subclones pMBG109, pMBG110, pMBG105, pMBG102, pMBG103, pMBG58, and pMBG57 after growth in low- and high-iron T media (Fig. 6). Alkaline phosphatase activity of CC118 containing  
10 pMBG109 was comparable to that of pMBG59, but alkaline phosphatase activity of all subclones having less than 1.5 kbp of chromosomal DNA upstream of the fusion joint was minimal. This amount of upstream chromosomal DNA is substantially more than that predicted to encode IrgA based on Western blot analysis.

15 (iv) Northern blot analysis of the irgA transcript. Northern blot analysis of the irgA transcript was performed to determine the size of the irgA transcript and to distinguish whether or not iron regulation of irgA occurs at the transcriptional level. RNA was prepared from strains MBG40 and 0395 following growth in low- and high-iron media.  
20 The blot was probed with the restriction fragment located between the HindIII and SmaI sites on the insert of pMBG59 (Fig. 6). A single band is seen at 2.2 kilobases (kb) in RNA from 0395 grown under low-iron conditions (Fig. 7, Lane 1b), and two less intense bands are seen at 2.5 and 2.1 kb in RNA from MBG40 grown under low-iron conditions (Lane  
25 2b). No bands are seen in either strain grown under high-iron conditions (Lanes 1a and 2a), demonstrating that iron regulation occurs at the transcriptional level. After DNA sequencing, these results were

- 26 -

confirmed by probing with a synthetic oligonucleotide shown to be entirely internal to irgA (data not shown).

To investigate the basis of the two bands seen in MBG40, we synthesized oligonucleotides complementary to phoA and to an open reading frame immediately downstream of phoA, as reported by Chang et al. [7]. Probing additional Northern blots with these two oligonucleotides demonstrated that the two bands seen in MBG40 are not a result of two transcriptional start sites for irgA, but a result of two downstream transcription termination sites within the TnphoA portion of the insert, one after phoA and the second located 400 bp further downstream, after the next open reading frame beyond phoA (data not shown). The 2.1 kb band from MBG40 is the transcript formed when termination occurs at a site located approximately 1.4 kb downstream from the fusion joint, and the 2.5 kb band is the transcript formed when termination occurs at a site approximately 1.8 kbp downstream from the fusion joint.

Comparison of the size of the IrgA'-PhoA fusion protein by Western blot analysis (70 kDa) with the size of the smaller transcript seen in MBG40 by Northern blot analysis (2.1 kb) suggests that the RNA transcript for irgA is monocistronic and has a transcription start site located near the 5' BglII restriction site in the DNA upstream of the fusion joint (Fig. 6). Localization of the transcription start site to this region was confirmed by probing Northern blots of MBG40 RNA with synthetic oligonucleotides complementary to DNA sequence located between the HindIII and upstream BglII restriction sites (irgA transcript not seen) and to DNA sequence located approximately 220 bp downstream of this BglII site (irgA transcript seen; data not shown).

- 27 -

These data, coupled with the alkaline phosphatase assays of the plasmid deletion subclones (Fig. 6), suggest that approximately 900 bp of DNA upstream of the predicted transcription start site is required for production of an active *lrgA'*-*PhoA* fusion protein.

5       (v) Northern blot analysis of *lrgA* transcription from plasmid deletion subclones. In order to determine whether the 900 bp region of DNA upstream of the predicted transcription start site is required for transcription of *lrgA* or for some step in post-transcriptional processing or activation of the fusion protein, we analysed RNA  
10       prepared from CC118 containing the plasmid deletion subclones in parallel with MBG40 RNA, following growth in low-iron media. The Northern blot was probed with the HindIII-SmaI restriction fragment used previously. Identical double bands, representing the two *lrgA* transcripts, were seen in lanes containing RNA from MBG40 and RNA from  
15       CC118 containing pMBG53, pMBG59, and pMBG109, but were absent in all other lanes (Fig. 6). Thus, plasmid deletion subclones having full iron-regulated alkaline phosphatase activity also had the *lrgA* transcripts, and those lacking alkaline phosphatase activity lacked the *lrgA* transcripts. The presence of approximately equal amounts of RNA  
20       in the lanes of this Northern blot was confirmed for the plasmid deletion subclones by reprobing the membrane with a restriction fragment internal to the ampicillin resistance gene from pBR322 (data not shown). These data suggest that the 900 bp of DNA upstream of the predicted transcription start site are necessary for transcription of  
25       *lrgA*, rather than for a post-transcriptional event. The fact that the *lrgA* transcripts seen in *E. coli* CC118 containing the active plasmid deletion subclones are identical to the transcripts seen with the

- 28 -

irgA::phoA fusion on the chromosome of V. cholerae strain MBG40 suggests that the results with CC118 (Fig. 6) are not a plasmid-related artifact nor unique to E. coli rather than V. cholerae.

DNA sequence of the irgA'-phoA fusion and upstream DNA. Figure 8 (corresponding to SEQ ID NO.: 1)

shows the DNA sequence of the chromosomal insert of pMBG59 from the

ClaI site to the fusion with phoA. A 453 bp open reading frame, which

is in-frame with phoA, begins at position 1077, representing the 5'

portion of irgA. A Shine-Dalgarno sequence is located just upstream of

the initiating methionine. The size of irgA upstream of the fusion

joint with phoA is slightly smaller than we had predicted by Western

blot of IrgA'-PhoA and by Northern blot of the irgA transcript, but

within reasonable experimental error.

The 900 bp region of DNA upstream of the BglII site that is

required for transcription of irgA includes an 894 bp open reading

frame in inverse orientation to irgA (Fig. 6). This open reading frame

begins at position 932, 144 bp upstream of the irgA open reading frame,

and terminates at position 39, just downstream of the ClaI restriction

site.

Primer extension analysis of the start site of irgA transcription.

Primer extension analysis of RNA from MBG40 grown in low iron was done

using two distinct synthetic oligonucleotides complementary to DNA

sequences located 20 bases and 64 bases downstream of the methionine

start codon (data not shown). Both oligonucleotides identified the

same approximate transcription start site, which is indicated by an

asterisk in Figure 8. A -10 box, reasonably homologous to the E. coli

consensus sequence [14], was located approximately 7 bases upstream of

the transcription start site, but no consensus -35 box was identified.

- 29 -

A 19 bp interrupted dyad symmetric sequence, homologous to the Fur binding consensus sequence of *E. coli* [5,6], was located immediately downstream of the transcription start site (Fig. 9: indicated by converging horizontal arrows in Fig.8).

5        IrgA protein analysis. The predicted amino acid sequence encoded by the portion of irgA located upstream of the fusion joint with phoA is shown in Figure 8.(corresponding to SEQ ID NO.: 1).

10        (i) Hydropathicity index. The hydropathicity index profile of this portion of IrgA is shown in Figure 10 (a positive index indicates hydrophobic residues and a negative index indicates hydrophilic residues). A stretch of hydrophobic residues, consistent with a signal sequence, is seen at the amino-terminus. This is followed by a second stretch of hydrophobic residues, 24 amino acids in length, suggestive of a possible transmembrane domain.

15        (ii) Homology of IrgA to E. coli ferrienterochelin receptor. The 151 amino acids at the amino-terminus of IrgA were analyzed using the FASTP algorithm for homologous proteins in the NBRF Protein Database. The best match in this search was the *E. coli* ferrienterochelin receptor, FepA, an 80 kDa iron-regulated outer membrane protein [20].  
20        The optimized score between the amino terminus of IrgA and FepA was 185. There were several regions of amino acid homology at roughly the same positions in each protein, including one stretch of ten consecutive identical amino acid residues (Fig.11).

25        Citrate utilization by NBG40. In addition to an iron assimilation system that utilizes the siderophore vibriobactin, *V. cholerae* has been shown to have a ferric citrate iron uptake system, enabling it to utilize citrate for growth in low iron media [34]. We therefore

- 30 -

evaluated whether or not MBG40 was able to utilize citrate to enhance growth in low-iron media and whether or not the expression of irgA, as determined by alkaline phosphatase assay, was altered by the addition of citrate. Cell density and alkaline phosphatase activity were measured for MBG40 after growth in low- and high-iron media with and without the addition of citrate (Table 4). The addition of citrate to iron-deficient media partially restores growth of the mutant, demonstrating that the mutant is able to utilize citrate. Further, alkaline phosphatase activity of the irgA'-'phoA fusion was not regulated in response to citrate. These data suggest that the ferric citrate iron uptake system is intact in the mutant MBG40 and that irgA is not involved in ferric citrate uptake.



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- 37 -

Example 3: Cloning and characterization of *irgB*, and insertional inactivation of *irgB* in *V. cholerae*

MATERIALS AND METHODS

Bacterial strains. The *V. cholerae* wild-type strain used in this study was classical Ogawa strain 0395 Sm<sup>r</sup>. *V. cholerae* strain MBG40 is 0395 *irgA*::Tnp<sub>phoA</sub>. *E. coli* strain CC118 is a *phoA* deletion derivative of MC1000 (13), and *E. coli* strains SY327  $\lambda$  *pir* and SM10  $\lambda$  *pir* have been described previously (14). *V. cholerae* strains MBG259 and MBG260 were constructed as described in Genetic Methods below.

Media. Two types of liquid media were used to evaluate the effect of iron concentration on gene expression: (i) LB medium with or without the addition of the iron chelator 2,2-dipyridyl (final concentration 0.2 mM), and (ii) tris-buffered medium (T medium) with or without the addition of FeSO<sub>4</sub> (final concentration 36  $\mu$ M). For *E. coli* strain CC118, T medium was supplemented with thiamine (10  $\mu$ g/ml) and the L-amino acids arginine and leucine (40  $\mu$ g/ml).

Construction of plasmids. Strain MBG40 contains a chromosomal gene fusion between *irgA* and *phoA*, constructed by Tnp<sub>phoA</sub> mutagenesis. Plasmids pMBG59, pMBG110, pMBG103, and pMBG58 contain subclones of the intact *irgA*::Tnp<sub>phoA</sub> gene fusion from MBG40 into plasmid pBR322, with decreasing amounts of chromosomal DNA upstream of *irgA* as described in Example 2. Plasmid pMBG59 contains all of the open reading frame upstream of *irgA* (subsequently named *irgB*, see Results below), while plasmids pMBG110, pMBG103, and pMBG58 contain progressively less upstream DNA and larger deletions of *irgB* (Fig. 6).

Plasmids pSBC45 and pSBC46 are derivatives of pACYC184, a plasmid encoding chloramphenicol resistance,

- 38 -

which is compatible with pBR322 and its derivatives. Plasmids pSBC45 and pSBC46 were constructed by isolating the NruI-SmaI fragment of plasmid pMBG59 by electroelution from a gel and ligating it into NruI digested pACYC184; the two plasmids differ only in the orientation of the inserted fragment. The NruI-SmaI fragment of pMBG59 contains the intact gene irgB (Fig. 6, see Results below).

Plasmid pMBG111 was derived from pGP704, a broad host range plasmid containing the ampicillin resistance gene from pBR322, the mobilization domain of plasmid RP4 (15), the origin of replication from plasmid R6K (16), and a polylinker from M13 tg131 (Amersham Corp.). Plasmid pGP704 was a gift of Gregory D. N. Pearson and is itself derived from plasmid pJM703.1 (14). Plasmid pGP704 and its derivatives are able to replicate only in strains containing the pir gene, which encodes the  $\pi$  protein necessary for the function of the R6K origin (16). To construct pMBG111, a 676 bp HincII-BglIII fragment of pMBG59 internal to irgB (Fig. 6) was ligated into the EcoRV and BglIII sites of the pGP704 polylinker.

Genetic methods. V. cholerae strains MBG259 and MBG260, which contain insertion mutations in irgB, were constructed from strains 0395 and MBG40, respectively, in the following manner. Plasmid pMBG111 was transferred from strain SY327  $\lambda$  pir into SM10  $\lambda$  pir by transformation. SM10  $\lambda$  pir contains a chromosomally-integrated RP4-2 (Tc::Mu), which encodes trans-acting factors necessary to mobilize pGP704 derivatives into a broad range of recipients without RP4 itself being transferred (14). SM10  $\lambda$  pir containing pMBG111 was conjugated separately with 0395 and MBG40, with double selection for ampicillin resistance (encoded by pMBG111) and streptomycin resistance (encoded by 0395 or MBG40). Because 0395 and MBG40 do not contain the pir gene,



- 39 -

pMBG111 is unable to replicate in either of these strains, so that doubly-resistant colonies arise by homologous recombination between the internal fragment of irgB on pMBG111 and the corresponding chromosomal gene on the recipient, causing insertional inactivation of irgB. To confirm that chromosomal integration occurred within irgB on the recipient, we performed Southern hybridization of chromosomal DNA digests using EcoRV, an enzyme that does not cut within either irgB or pMBG111. The blot was probed with the HincII-BglII fragment of irgB, radioactively labeled by random primer extension with a commercial kit (Prime Time, International Biotechnologies, Inc.).

Plasmids were transformed into E. coli strains by standard techniques (17). Plasmids pACYC184 and pSBC45 were introduced into V. cholerae strains 0395, MBG40, MBG259, and MBG260 by electroporation, using the protocol of the manufacturer (Gene Pulser, Bio-Rad, Rockville Centre, NY), with the exception of substitution of 2mM  $\text{CaCl}_2$  as the buffer for resuspending cells during preparation, rather than water or HEPES buffer.

Assays. The enzymatic activity of alkaline phosphatase encoded on Tnp<sub>phoA</sub> permitted the comparison of fusion gene expression when strains were grown in low- versus high-iron media. Strains were grown overnight in either LB medium with or without added 2,2-dipyridyl or T medium with or without added  $\text{FeSO}_4$ . Alkaline phosphatase activity was determined as described in Example 1.

DNA and RNA analysis. Analysis of DNA and RNA, including DNA and RNA preparation, restriction mapping, DNA sequencing, Northern blot analysis and primer extension were performed as described in Example 2. For Northern blot analysis, an equivalent quantity of RNA, as calculated from  $\text{OD}_{260}$ , was loaded into each lane.

- 40 -

Synthetic oligonucleotides used as probes for Northern blot analysis and as primers for DNA sequencing and primer extension were the generous gift of Brian Seed.

5        Protein analysis and protein database searches.

Protein analysis and protein database searches were performed using IBI-Pustell Sequence Analysis software (International Biotechnologies, Inc., New Haven, CT.). The hydropathicity index profile of IrgB was calculated  
10 by the formula of Kyte-Doolittle (18). The protein secondary structure prediction of IrgB was calculated by the algorithm of Chou-Fasman (19). Database searches and protein alignments were performed by searching the NBRF Protein Database (Release 19) using the FASTP algorithm  
15 for protein homology (20).

RESULTS

Northern blot analysis of the open reading frame upstream of irgA. In order to determine whether an RNA transcript was associated with the upstream open reading  
20 frame, and if present, to determine the size of the transcript and whether or not transcription was regulated by iron, we performed Northern blot analysis of RNA prepared from strains 0395 and MBG40 following growth in low- and high-iron media (Fig. 12). The blot was probed  
25 with an oligonucleotide complementary to the DNA sequence near the 5' terminus of the open reading frame. A single band of approximately 1.1 kilobases (kb) in size was seen in RNA prepared from 0395 and MBG40 grown under low-iron conditions (Fig. 12, Lanes 2 and 4), demonstrating that a  
30 transcript was associated with the open reading frame. No bands were seen in either strain grown under high-iron conditions (Fig. 12, Lanes 1 and 3), demonstrating that transcription of the open reading frame was negatively regulated by iron. The size of the

- 41 -

transcript, in relation to the size of the open reading frame (894 bp, see below), suggests that the transcript is monocistronic. The iron-regulated gene encoded by this open reading frame was designated irgB.

5        Trans-complementation of irgB and irgA. We have previously demonstrated, by deletion subcloning of pMBG59 in an E. coli background, that deletion of any portion of irgB eliminates transcription of irgA (Example 2). We wished to examine whether irgB could restore expression  
10 of irgA in trans.

(i) Trans-complementation in an E. coli background. Alkaline phosphatase assays were performed for E. coli strain CC118 containing plasmid pMBG59 and plasmid deletion subclones pMBG110, pMBG103, and pMBG58,  
15 alone and in combination with pSBC45 (which carries an intact irgB gene) following growth in low- and high-iron media (Table 5). CC118 containing pMBG59, which carries all of irgB and the irgA'-'phoA fusion, had significant iron-regulated alkaline phosphatase activity, as  
20 described in Example 2. CC118 containing pMBG110, pMBG103, or pMBG58, each with a progressive deletion of irgB (Fig. 6), had lost alkaline phosphatase activity that could be partially or completely restored by pSBC45 in trans. Restoration of iron regulation by pSBC45 in  
25 trans was variable or incomplete in the E. coli background with high copy number plasmids. Complementation of pMBG110, pMBG103, or pMBG58 with the vector plasmid pACYC184 in CC118 did not restore alkaline phosphatase activity (data not shown). In addition,  
30 alkaline phosphatase activities of strains complemented with pSBC46 (the intact irgB gene cloned in the opposite orientation in pACYC184) were similar to those obtained for strains complemented with pSBC45 (data also not shown).

- 42 -

(ii) Trans-complementation in *V. cholerae*. In order to examine trans-complementation of *irgB* and *irgA* in the *V. cholerae* background, we constructed the *irgB* mutant strains MBG260 and MBG259 from MBG40 and 0395 respectively. Highly expressed, iron-regulated alkaline phosphatase activity seen in strain MBG40 was almost completely eliminated with the introduction of the *irgB* mutation to make strain MBG260 (Table 5). Iron-regulated alkaline phosphatase activity was completely restored by the introduction of *irgB* in trans on plasmid pSBC45 (Table 5), while the introduction of the vector pACYC184 had no effect (data not shown). Strains 0395 and MBG259, which do not contain an *irgA*'-'*phoA* fusion, had negligible alkaline phosphatase activity, with or without the introduced plasmids (data not shown). The higher alkaline phosphatase activities seen in MBG260(pSBC45) and MBG40(pSBC45), as compared to MBG40 (Table 5), may result from the high copy number of *irgB* carried on pSBC45. These data suggest that *irgB* is a trans-acting factor that positively regulates *irgA*.

DNA sequence and deduced protein sequence of *irgB*.

Fig. 13 (SEQ ID NO.: 2) shows the DNA sequence of the chromosomal insert of pMBG59 (reading 5' to 3' from right to left in Fig. 6), starting approximately 60 bp beyond the transcription start site of *irgA* and extending up to the *ClaI* site of pMBG59, including the 894 bp open reading frame of *irgB*. A Shine Dalgarno sequence is indicated just upstream of the initiating methionine (21). A perfect inverted repeat, suggestive of a transcription terminator, is indicated just beyond the termination codon of the *irgB* open reading frame.

Primer extension analysis of the start site of *irgB* transcription. Primer extension analysis of RNA prepared from MBG40 and 0395 following growth in low-iron media was done using a synthetic oligonucleotide

- 43 -

complementary to the DNA sequence located between 3 bases upstream and 17 bases downstream of the methionine start codon (data not shown). The same transcription start site was identified in both MBG40 and 0395 and is indicated by an asterisk in Figure 13. A promoter homologous to the E. coli consensus sequence (22) was located upstream of the transcription start site (Fig. 13; SEQ ID NO.: 2). The 19 bp interrupted dyad symmetric sequence that is homologous to the Fur binding consensus sequence of E. coli (5,23), and is located immediately downstream of the irgA transcription start site, also overlaps the irgB transcription start site and -10 box (Fig. 13; SEQ ID NO.: 2). Fig. 14 shows the overlapping but divergent irgA and irgB promoters, and the location of the Fur-like box in relation to each.

IrgB protein analysis. The predicted amino acid sequence of IrgB is shown below the nucleotide sequence in Fig. 13 (SEQ ID NO.: 2).

(i) Hydropathicity profile. The hydropathicity profile of IrgB showed no stretches of hydrophobic residues that would be suggestive of either a signal sequence or a transmembrane domain (data not shown).

(ii) Homology of IrgB to the LysR family of positive transcriptional activators. Comparison of IrgB to the NBRF Protein Database demonstrated significant homology between IrgB and the LysR family of positive transcriptional activator proteins in bacteria (24). The best match in this family was to the E. coli positive activator protein IlvY; the optimized score between IrgB and IlvY was 217. The regions of highest homology between IrgB and the LysR family were near the amino terminus and were at roughly the same positions in each protein. The homology near the amino terminus of IrgB with several members of the LysR family is shown in Fig. 15. All of the proteins shown are of approximately the

- 44 -

same size (IrgB is 298 amino acids; IlvY, LysR, AmpR, NodD, and CysB are 297, 300, 291, 300, and 301 amino acids, respectively).

(iii) Prediction of the secondary structure of

- 5 IrgB. The secondary structure of IrgB was predicted using the Chou-Fasman algorithm (19). A helix-turn-helix motif was seen in the same region of IrgB as in the other members of the LysR family (Fig. 15) (24).

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TABLE 1

TABLE 1. Alkaline phosphatase assays in LB medium  
with and without added dipyrldyl

	Strain	Alkaline phosphatase activity (U <sub>A<sub>540</sub>)</sub>		Induction ratio
		Without dipyrldyl	With dipyrldyl	
5	0395	1	1	1
	MBG13	2	773	387
	MBG14	1	50	50
10	MBG15	1	689	689
	MBG17	3	1.147	382
	MBG18	3	39	13
	MBG19	1	113	113
	MBG20	1	268	268
15	MBG21	1	259	259
	MBG22	1	338	338
	MBG23	1	386	386
	MBG24	1	370	370
	MBG25	1	353	353
20	MBG26	1	300	300
	MBG27	6	344	57
	MBG28	1	620	620
	MBG29	1	318	318
	MBG30	1	437	437
25	MBG31	3	72	24
	MBG33	1	136	136
	MBG34	1	86	86
	MBG35	1	25	25
	MBG37	2	197	99
30	MBG38	100	575	6
	MBG39	4	39	10
	MBG40	1	857	857

TABLE 2

TABLE 2. Virulence assays of wild-type and mutant strains

Strain	Competing strain	Competitive index		LD <sub>50</sub> (no. of bacteria)
		In vitro	In vivo	
0395				$4 \times 10^3$
MBG40	0395	0.98	0.11	$3 \times 10^3$

TABLE 3 Alkaline phosphatase assays in T media with and without added iron.

5	Strain	Alkaline phosphatase activity (U/A <sub>600</sub> )	
		With iron	Without iron
10	0395 (pSBC34)	82	496
	CC118 (pMBC53)	63	277

TABLE 4 Growth and alkaline phosphatase activity of MBG40  
grown overnight in low and high iron T media. with and  
without added citrate.

Growth conditions	A <sub>600</sub>	Alkaline phosphatase activity (U/A <sub>600</sub> )
Low iron, without citrate	0.397	320
Low iron, with citrate	0.801	167
High iron, without citrate	1.431	1
High iron, with citrate	1.663	1

TABLE 5. Complementation of irgB and irgA'-phoA  
in E. coli and V. cholerae.

Alkaline phosphatase assays in low and high iron media.

Strain	Alkaline phosphatase activity (U/A <sub>600</sub> )	
	Low iron	High iron
Complementation in <u>E. coli</u>		
CC118(pMBG59)	222	60
CC118(pMBG110)	4	1
CC118(pMBG103)	3	1
CC118(pMBG58)	1	1
CC118(pSBC45)	0	1
CC118(pMBG110, pSBC45)	209	11°
CC118(pMBG103, pSBC45)	124	68
CC118(pMBG58, pSBC45)	63	108
Complementation in <u>V. cholerae</u>		
MBG40	683	3
MBG260	4	1
MBG40(pSBC45)	1420	11
MBG260(pSBC45)	1573	8

Assays of E. coli strains were performed following growth in T medium with and without added FeSO<sub>4</sub>; assays of V. cholerae strains were performed following growth in LB medium with and without added dipyrldyl.

- 52 -

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

Goldberg, Marcia  
Calderwood, Stephen B.  
Mekalanos, John J.

(ii) TITLE OF INVENTION: *VIBRIO CHOLERAE* STRAINS  
DEFECTIVE IN *irgA*  
EXPRESSION, AND CHOLERA  
VACCINES DERIVED  
THEREFROM

(iii) NUMBER OF SEQUENCES:

2

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(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage  
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX  
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 3.30)  
(D) SOFTWARE: WordPerfect (Version 5.0)

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 07/629,102  
(B) FILING DATE: December 18, 1990  
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## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1530  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATCGATGATA AAAAATCCCG CTGCGGCGGG ATTTTTTATT GCCACTCATC GGGCCTTGCT	60
TGGCGGAGCG CATCAATAAA TAGGCGCAGC CGAAGTGGGT GACGACCGAG CGGATAGAAG	120
CAGTTGATTT CTGTTGGCTG TGATTGCCAT CCGTTGACGC AAGGAATGAG GCTGCCCCGA	180
TGCGCCGTTT CAAAACCATT GGCAAACCAA GTGGGAAGCA AACCAATACC ACGACCTTTA	240
GCAATCGCAT CGGCTTG CAT GGCAAGATTA TCGCTTTGTA AACGACTCTC TAGTGCTGGC	300
AGTGAATAAC TGCCGAACTC TGGATGGTGC AGTTCAAGCT CCGCGCGCCG ACAAGCAATA	360
AAATCAATCC ATGGGTGATG AATCAGCTCA CGAGGATGGG TCGGTTTATC TCGATGGGCC	420
AAATATTTGG GAGAGGCGTA AGTGGCATAG CGCCAATAGC CTAAGCGTTC TTTGCGATAA	480
CCCATGGGGG CGGCGTGTTT AATCCAAATG ATCAAATCGG GCTCAAACAC CTCATCACTG	540
TGTTGAAACT GGCTGAGTAG ACGGATCTTC AATGTGCAAT GCTGCTGCAT AAATCATCC	600
AATACTTGGC TGAGCCAGCC GCGGATCAAA TTGGGGTGTA CCACCAGCGT GAGTTCGCCA	660
GTCACCTGAT TGTTC AATC TTGCAACGCT TCCTGACTTT TATTGGCCAG TTCAAGTAGT	720
TGCTCCGAGT AAACCGCAAA CACTTCTCCT GCTTTGGTGA GCGTTAAGCG GTTGCCTTGA	780
CGCATCAACA AGCTTTGTCC CAAGTCCTCT TCAAGTTGCG CCAAACGGCG ACTCAGGGTG	840
GATTTAGGCT GTTCAAGCGC TTTGGCAGCG GCAGTCAGGC TCTTATGTTG GCAAAGCGCA	900
TGGAAAGCTT TTACGGCGCT GAGATCTTGC ATAGGTATTT GACCCTTAAA GAATAATTAC	960
CACAGACGTT CCATATTTGG ACCGAACTAT TCCATGTGTC GATCTATCTC CAGTACAGAA	1020
TATATGAATA ATCCGCTTCT GAAATTAAGA ATAATTATCA TTAAAGGAG TGGTAA ATG	1079
Met	
1	
TCC AGA TTC AAT CCA TCC CCC GTC AGT TTA TCT GTG ACA CTA GGC TTA	1127

- 54 -

Ser Arg Phe Asn Pro Ser Pro Val Ser Leu Ser Val Thr Leu Gly Leu  
 5 10 15  
 ATG TTT TCG GCT AGC GCT TTT GCT CAA GAC GCG ACG AAA ACG GAT GAA  
 Met Phe Ser Ala Ser Ala Phe Ala Gln Asp Ala Thr Lys Thr Asp Glu  
 20 25 30 1175  
 ACC ATG GTG GTC ACT GCG GCG GGA TAC GCG CAA GTG ATT CAA AAT GCA  
 Thr Met Val Val Thr Ala Ala Gly Tyr Ala Gln Val Ile Gln Asn Ala  
 35 40 45 1223  
 CCA GCC AGT ATC AGT GTG ATT TCA AGA GAA GAT CTG GAA TCT CGC TAT  
 Pro Ala Ser Ile Ser Val Ile Ser Arg Glu Asp Leu Glu Ser Arg Tyr  
 50 55 60 65 1271  
 TAC CGT GAT GTG ACC GAT GCG CTA AAA AGC GTA CCG GGT GTG ACA GTC  
 Tyr Arg Asp Val Thr Asp Ala Leu Lys Ser Val Pro Gly Val Thr Val  
 70 75 80 1319  
 ACC GGA GGG GGC GAT ACT ACC GAT ATC AGC ATT CGT GGT ATG GGA TCA  
 Thr Gly Gly Gly Asp Thr Thr Asp Ile Ser Ile Arg Gly Met Gly Ser  
 85 90 95 1367  
 AAC TAT ACT CTT ATC TTG GTG GAT GGT AAG CGC CAA ACC TCA CGC CAG  
 Asn Tyr Thr Leu Ile Leu Val Asp Gly Lys Arg Gln Thr Ser Arg Gln  
 100 105 110 1415  
 ACC CGT CCA AAC AGC GAT GGC CCG GGC ATT GAG CAA GGT TGG TTA CCG  
 Thr Arg Pro Asn Ser Asp Gly Pro Gly Ile Glu Gln Gly Trp Leu Pro  
 115 120 125 1463  
 CCA CTG CAA GCG ATT GAA CGT ATC GAG GTG ATC CGT GGC CCG ATG TCT  
 Pro Leu Gln Ala Ile Glu Arg Ile Glu Val Ile Arg Gly Pro Met Ser  
 130 135 140 145 1511  
 ACG CTG TAC GGC TCG GAT G  
 Thr Leu Tyr Gly Ser Asp  
 150 1530



- 55 -

(2) INFORMATION FOR SEQ ID NO: 2:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1100  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGGGGATGGA TTGAATCTGG ACATTTACCA CTCCTTTAAA TGATAATTAT TCTTAATTTT 60  
 AGAAGCGGAT TATTCATATA TTCTGTACTG GAGATAGATC GACACATGGA ATAGTTCGGT 120  
 CCAAATATGG AACGTCTGTG GTAATTATTC TTAAAGGGTC AAATACCT ATG CAA GAT 177  
 Met Gln Asp  
 1  
 CTC AGC GCC GTA AAA GCT TTC CAT GCG CTT TGC CAA CAT AAG AGC CTG 225  
 Leu Ser Ala Val Lys Ala Phe His Ala Leu Cys Gln His Lys Ser Leu  
 5 10 15  
 ACT GCC GCT GCC AAA GCG CTT GAA CAG CCT AAA TCC ACC CTG AGT CGC 273  
 Thr Ala Ala Ala Lys Ala Leu Glu Gln Pro Lys Ser Thr Leu Ser Arg  
 20 25 30  
 CGT TTG GCG CAA CTT GAA GAG GAC TTG GGA CAA AGC TTG TTG ATG CGT 321  
 Arg Leu Ala Gln Leu Glu Glu Asp Leu Gly Gln Ser Leu Leu Met Arg  
 40 45 50  
 CAA GGC AAC CGC TTA ACG CTC ACC AAA GCA GGA GAA GTG TTT GCG GTT 369  
 Gln Gly Asn Arg Leu Thr Leu Thr Lys Ala Gly Glu Val Phe Ala Val  
 55 60 65  
 TAC TCG GAG CAA CTA CTT GAA CTG GCC AAT AAA AGT CAG GAA GCG TTG 417  
 Tyr Ser Glu Gln Leu Leu Glu Leu Ala Asn Lys Ser Gln Glu Ala Leu  
 70 75 80  
 CAA GAA TTG AAC AAT CAA GTG ACT GGC GAA CTC ACG CTG GTG GTA CAC 465  
 Gln Glu Leu Asn Asn Gln Val Thr Gly Glu Leu Thr Leu Val Val His  
 85 90 95  
 CCC AAT TTG ATC CGC GGC TGG CTC AGC CAA GTA TTG GAT GAG TTT ATG 513  
 Pro Asn Leu Ile Arg Gly Trp Leu Ser Gln Val Leu Asp Glu Phe Met  
 100 105 110 115  
 CAG CAG CAT TCG ACA TTG AAG ATC CGT CTA CTC AGC CAG TTT CAA CAC 561  
 Gln Gln His Ser Thr Leu Lys Ile Arg Leu Leu Ser Gln Phe Gln His  
 120 125 130  
 AGT GAT GAG GTG TTT GAG CCC GAT TTG ATC ATT TGG ATT GAA CAC GCC 609  
 Ser Asp Glu Val Phe Glu Pro Asp Leu Ile Ile Trp Ile Glu His Ala

- 56 -

135	140	145	
GCC CCC ATG GGT TAT CGC AAA GAA CGC TTA GGC TAT TGG CGC TAT GCC Ala Pro Met Gly Tyr Arg Lys Glu Arg Leu Gly Tyr Trp Arg Tyr Ala 150 155 160			657
ACT TAC GCC TCT CCC AAA TAT TTG GCC CAT CGA GAT AAA CCG ACC CAT Thr Tyr Ala Ser Pro Lys Tyr Leu Ala His Arg Asp Lys Pro Thr His 165 170 175			705
CCT CGT GAG CTG ATT CAT CAC CCA TGG ATT GAT TTT ATT GCT TGT CGG Pro Arg Glu Leu Ile His His Pro Trp Ile Asp Phe Ile Ala Cys Arg 180 185 190 195			753
CGC GCG GAG CTT GAA CTG CAC CAT CCA GAG TTC GGC AGT TAT TCA CTG Arg Ala Glu Leu Glu Leu His His Pro Glu Phe Gly Ser Tyr Ser Leu 200 205 210			801
CCA GCA CTA GAG AGT CGT TTA CAA AGC GAT AAT CTT GCC ATG CAA GCC Pro Ala Leu Glu Ser Arg Leu Gln Ser Asp Asn Leu Ala Met Gln Ala 215 220 225			849
GAT GCG ATT GCT AAA GGT CGT GGT ATT GGT TTG CTT CCC ACT TGG TTT Asp Ala Ile Ala Lys Gly Arg Gly Ile Gly Leu Leu Pro Thr Trp Phe 230 235 240			897
GCC AAT GGT TTT GAA ACG GCG CAT CCG GGC AGC CTC ATT CCT TGC GTC Ala Asn Gly Phe Glu Thr Ala His Pro Gly Ser Leu Ile Pro Cys Val 245 250 255			945
AAC GGA TGG CAA TCA CAG CCA ACA GAA ATC AAC TGC TTC TAT CCG CTC Asn Gly Trp Gln Ser Gln Pro Thr Glu Ile Asn Cys Phe Tyr Pro Leu 260 265 270 275			993
GGT CGT CAC CCA CTT CGG CTG CGC CTA TTT ATT GAT GCG CTC CGC CAA Gly Arg His Pro Leu Arg Leu Arg Leu Phe Ile Asp Ala Leu Arg Gln 280 285 290			1041
GCA AGG CCC GAT GAG TGG CAA TAA AAAATCCCGC CGCAGCGGGA TTTTATCA Ala Arg Pro Asp Glu Trp Gln 295			1095
TCGAT			1100

- 57 -

CLAIMS

- 1 1. A *V. cholerae* cell harboring a mutation  
2 which inhibits expression in said cell of a functional  
3 *irgA* gene product.
- 1 2. The cell of claim 1, wherein said mutation  
2 prevents said expression.
- 1 3. The cell of claim 1, wherein said mutation  
2 is in said cell's *irgA* gene.
- 1 4. The cell of claim 3, wherein said mutation  
2 is a deletion.
- 1 5. The cell of claim 4, wherein at least 25% of  
2 the coding sequence of said *irgA* gene has been deleted.
- 1 6. The cell of claim 1, wherein said mutation  
2 is in said cell's *irgB* gene.
- 1 7. The cell of claim 6, wherein said mutation  
2 is a deletion.
- 1 8. The cell of claim 7, wherein at least 25% of  
2 the coding sequence of said *irgB* gene has been deleted.
- 1 9. A substantially purified preparation of *V.*  
2 *cholerae* cells, wherein each such cell harbors a mutation  
3 which inhibits expression in said cell of a functional  
4 *irgA* gene product.
- 1 10. The preparation of claim 9, wherein said  
2 mutation prevents said expression.

- 58 -

1 11. The preparation of claim 9, wherein said  
2 mutation is in said cell's *irgA* gene.

1 12. The preparation of claim 11, wherein said  
2 mutation is a deletion.

1 13. The preparation of claim 12, wherein at  
2 least 25% of the coding sequence of said *irgA* gene has  
3 been deleted.

1 14. The preparation of claim 9, wherein said  
2 mutation is in said cell's *irgB* gene.

1 15. The preparation of claim 14, wherein said  
2 mutation is a deletion.

1 16. The preparation of claim 15, wherein at  
2 least 25% of the coding sequence of said *irgB* gene has  
3 been deleted.

1 17. A cholera vaccine comprising live  
2 attenuated *V. cholerae* cells, each of which cells harbors  
3 a mutation which inhibits expression in said cell of a  
4 functional *irgA* gene product.

1 18. The cholera vaccine of claim 17, wherein  
2 said mutation prevents said expression.

1 19. The cholera vaccine of claim 17, wherein  
2 said mutation is a deletion in said cell's *irgA* gene.

1 20. The cholera vaccine of claim 17, wherein  
2 said mutation is a deletion in said cell's *irgB* gene.

- 59 -

1           21. The cell of claim 1, wherein said cell  
2 harbors a second mutation which inhibits production of a  
3 second *V. cholerae* virulence factor in said cell.

1           22. The cell of claim 21, wherein said second  
2 mutation prevents said production.

1           23. The cell of claim 22, wherein said second  
2 virulence factor is the A subunit of cholera toxin and  
3 said first and second mutations are deletions.

1           24. The cell of claim 23, wherein said first  
2 mutation is in said cell's *irgA* gene.

1           25. The cell of claim 23, wherein said first  
2 mutation is in said cell's *irgB* gene.

1           26. The preparation of claim 9, wherein each of  
2 said cells harbors a second mutation which inhibits  
3 production of a second *V. cholerae* virulence factor in  
4 said cell.

1           27. The preparation of claim 26, wherein said  
2 second mutation prevents said production.

1           28. The preparation of claim 26, wherein said  
2 second virulence factor is the A subunit of cholera toxin  
3 and said first and second mutations are deletions.

1           29. The preparation of claim 28, wherein said  
2 first mutation is in said cell's *irgA* gene.

1           30. The preparation of claim 28, wherein said  
2 first mutation is in said cell's *irgB* gene.

- 60 -

1           31. The cholera vaccine of claim 17, wherein  
2 each of said cells harbors a second mutation which  
3 inhibits production of a second *V. cholerae* virulence  
4 factor in said cell.

1           32. The cholera vaccine of claim 31, wherein  
2 said second mutation prevents said production.

1           33. The cholera vaccine of claim 32, wherein  
2 said second virulence factor is the A subunit of cholera  
3 toxin and said first and second mutations are deletions.

1           34. The cholera vaccine of claim 31, wherein  
2 said first mutation is in said cell's *irgA* gene.

1           35. The cholera vaccine of claim 31, wherein  
2 said first mutation is in said cell's *irgB* gene.

1           36. A cell derived from *V. cholerae* strain  
2 0395-N1, wherein said cell harbors a deletion mutation  
3 which inhibits the expression in said cell of an active  
4 *irgA* gene product.

1           37. A cholera vaccine comprising the cell of  
2 claim 36.

1           38. A method for inducing immunity to cholera  
2 in a mammal, which method comprises administering to said  
3 mammal an effective amount of the cholera vaccine of  
4 claim 31.

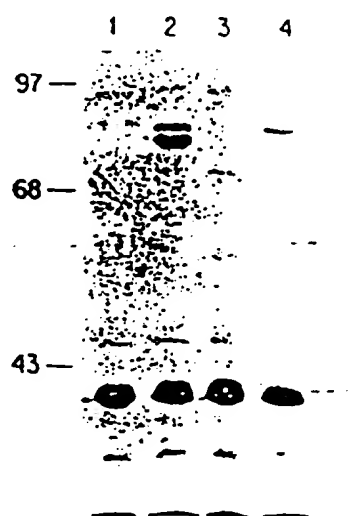
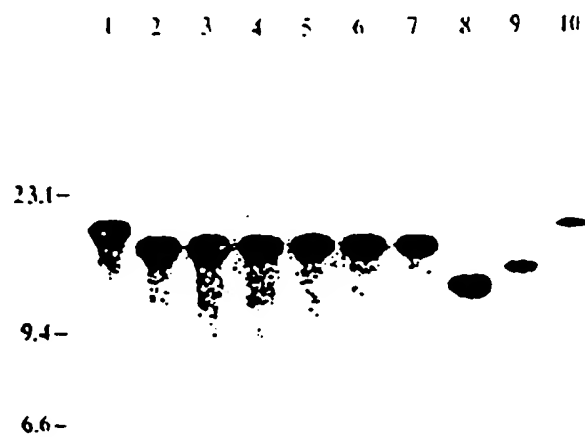
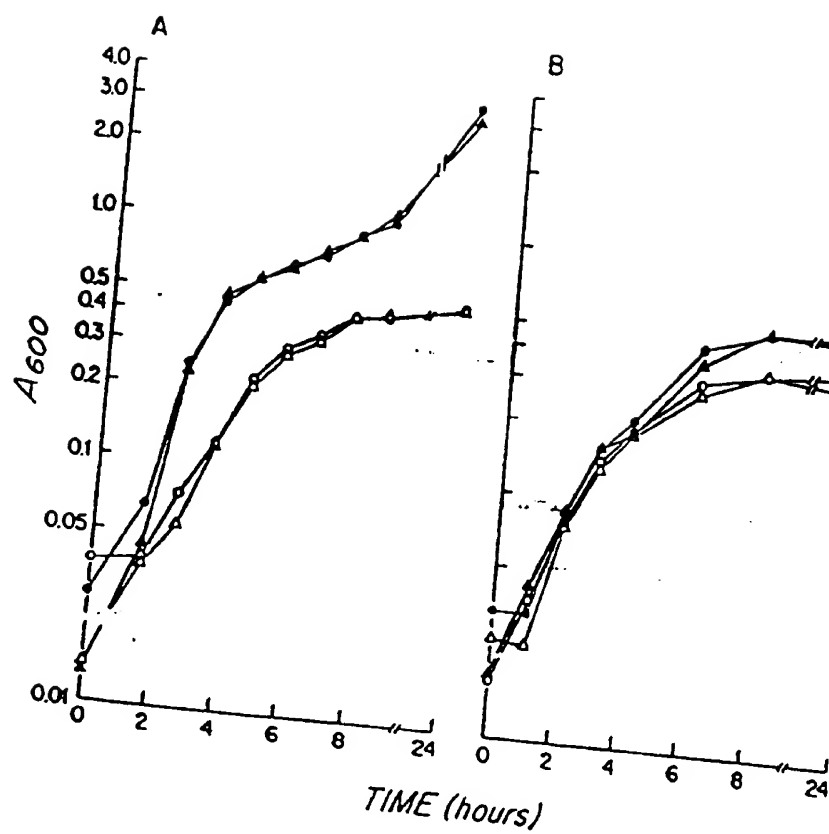
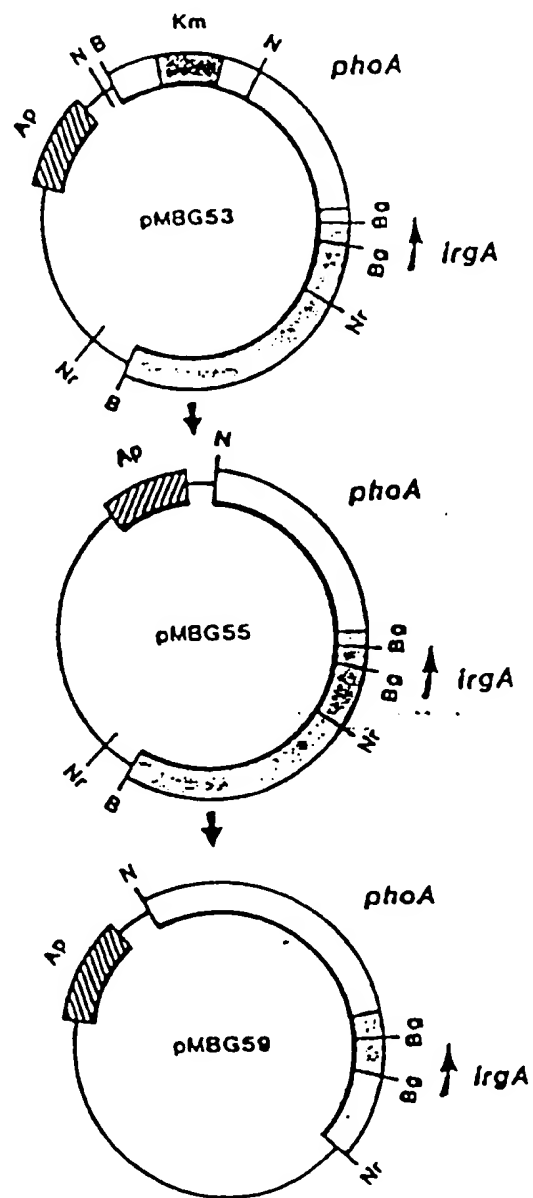


FIG. 2









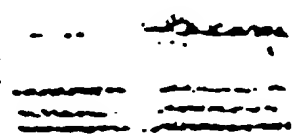
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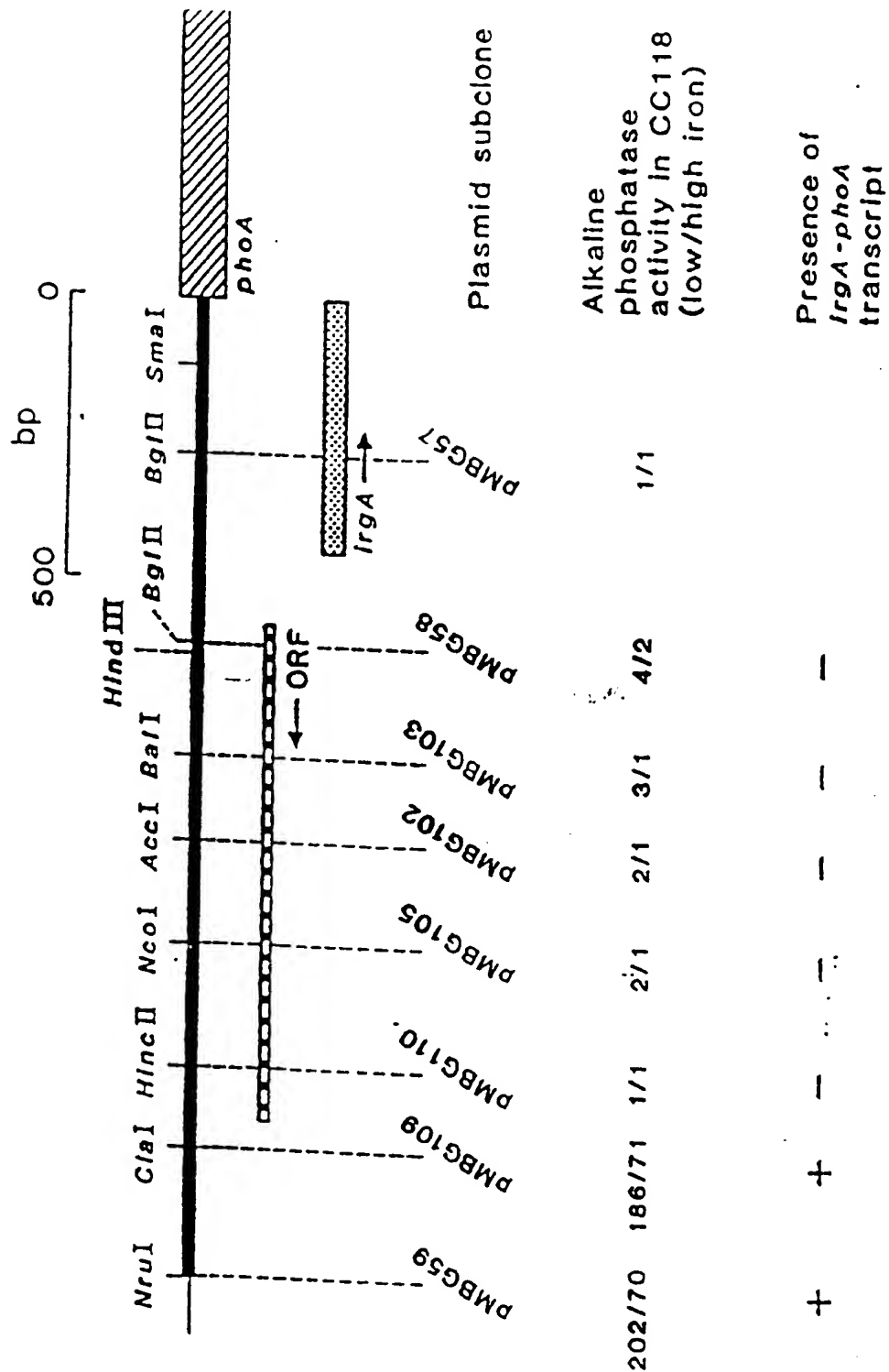
95-

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43-

36-





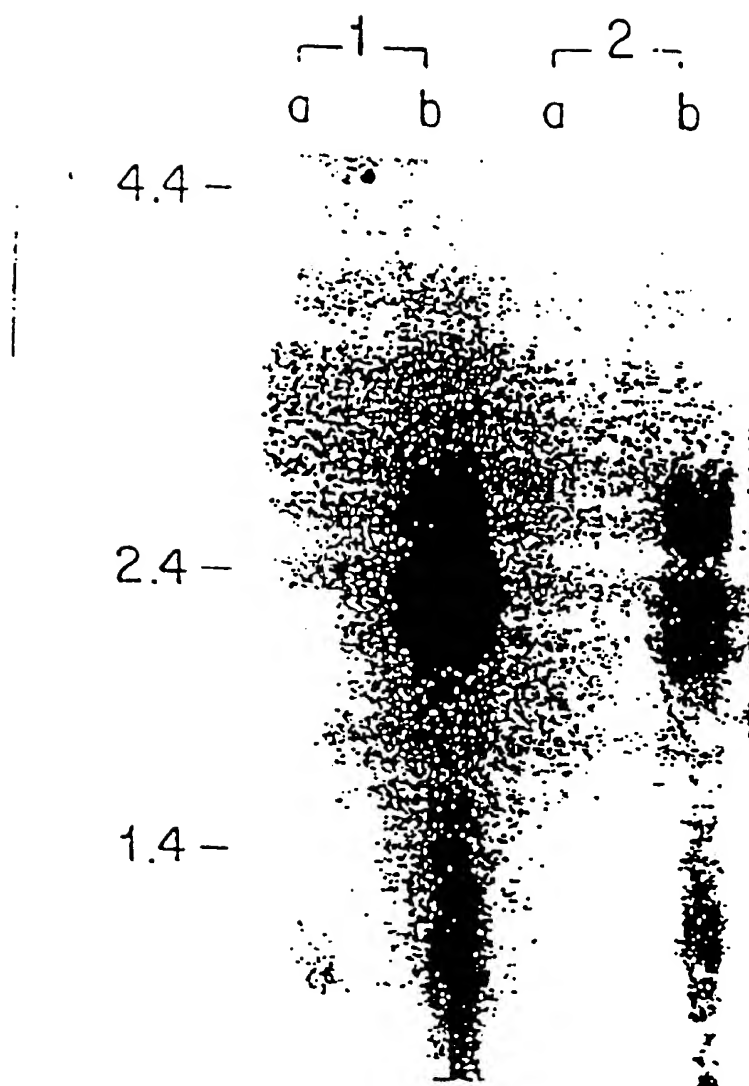


FIG. 8

Cla 10 20 30 40 50 60 70  
ATCATGATAAAATGGGTCTGGGGGCATTTTTATTCAGCACTATCGGGCTCTCTGGGAGCC

80 90 100 110 120 130 140  
CATCADAATACGGGGCGGAAGTGGCTACCAAGCAAGGCAATACAAGCGTTCAATTCTTGTCCTG

150 MindII 170 180 190 200 210  
TGATTGCCATGGCTTTCAGCGAAGCATGAGCTGCGGGGATGGGGTTTTTAAGCATGGCAAGCAA

220 230 240 250 260 270 280  
GTGGAGCAGAACCAATACCAAGACTTTAGCAATCGCATGGCGTTGCATCGCAATATGGCTTTGTA

290 300 310 320 330 340 350  
AAGACTCTCTAGTCTGTGCACTCAATCACTCGGCACTCTGCATCTGCGCTTCAAGCTGGGGGGGG

360 NoelI 380 390 400 410 420  
AAGCAATATAATCATCTGCTGCATCACTCACTCAAGAGATGGCTGGCTTTATCTGCATGGGG

430 440 450 460 470 480 490  
AAATATTGGCAGCGGTGAGTGCATAGGGGCAATAGGCTTAGGCTTCTTTGGCAAGCAATGGGG

500 510 520 530 540 550 AccI  
CGGGCTTTCAATCAATGATCAATATGGGCTCAAGAGCTCTATCTCTGTCTTCAAGCTGGCTGAGC

570 580 590 600 610 620 630  
AAGCATCTCAATCTCAATCTGCTGCAATCACTCACTCAAGAGCTGGCTTTCAGCGGGGGGATCAAA

640 650 660 670 680 690 700  
TTGGGCTGACCGGGCGTCACTGGGGCTGACTTCAATCTTCAATCTTCAAGCTTCTGACTTTT

PallI 720 730 740 750 760 770  
TATTGGCGTTCAAGTGTGCTGCTGGAGTAAAGGGCAAGCTCTCTGCTCTTCTGAGCGGTAGCG

780 790 800 810 820 830 840  
GTTCGCTTCAAGCATCAAGAGCTTGTGGCGAGTCTCTTCACTTGGGGCAAGCGGAGCTAGGGTG

850 860 870 880 890 900 MindIII  
CATTTAGGCTCTTCAAGCGCTTTGGGGGGGCTGAGCGCTCTATCTTCAAGCGGGGATCAAGCTTT

920 PallII 930 940 950 960 970 980  
TTAGGGGGCTCACATCTTCAATAGCTATTTCAGCTTAAAGATATTCAGAGCTTCAATATTTCG

990 1000 1010 1020 1030 1040 1050  
AAGCACTATTCAGTGTGCACTATCTTCAAGTACAGATATATCATTAATGGGCTTCAATTAAGA

-10

1060 1070 1080 1090 1100  
ATAATATCATTTAAGAGCTGCTAA ATC TCC ACA TTC AAT CCA TCC CCC GTC AGT TTA  
SD Met Ser Arg Phe Asn Pro Ser Pro Val Ser Leu

1120 1130 1140 1150 1160  
TCT GTC ACA CTA GCC TTA ATC TTT TCG OCT ACC CCT TTT OCT CAA GAC CGC ACC  
Ser Val Thr Leu Gly Leu Met Phe Ser Ala Ser Ala Phe Ala Glu Asp Ala Thr

1170 1180 1190 1200 1210  
AAA AOC CAT CAA AOC ATG CTC CTC ACT CGC GCG ACA TAC CGC CAA GTG ATT CAA  
Lys Thr Asp Glu Thr Met Val Val Thr Ala Ala Gly Tyr Ala Glu Val Ile Glu

1230 1240 1250 PallII 1260 1270  
AAT CCA CCA CCC AGT ATC AGT GTC ATT TCA ACA CAA CAT CTC CAA TCT CCC TAT  
Asn Ala Pro Ala Ser Ile Ser Val Ile Ser Arg Glu Asp Leu Glu Ser Arg Tyr

1280 1290 1300 1310 1320  
TAC CCT CAT GTC ACC CAT CGC CTA AAA AGC GTA CGC CGT GTC ACA GTC ACC CGA  
Tyr Arg Asp Val Thr Asp Ala Leu Lys Ser Val Pro Gly Val Thr Val Thr Gly

1330 1340 1350 1360 1370  
CGC GCG CAT ACT ACC CAT ATC AGC ATT CGT CGT ATG CCA TCA AAC TAT ACT CTT  
Gly Gly Asp Thr Thr Asp Ile Ser Ile Arg Gly Met Gly Ser Asn Tyr Thr Leu

1390 1400 1410 1420 1430  
ATC TTG GTC CAT CGT AGC CGC CAA ACC TCA CGC CAG ACC CGT CCA AAC ACC CAT  
Ile Leu Val Asp Gly Lys Arg Glu Thr Ser Arg Glu Thr Arg Pro Asn Ser Asp

SmaI 1450 1460 1470 1480  
CGC CGC CGC ATT GAG CAA CGT TCG TTA CGC CCA CTG CAA CGC AAT GAA CGT ATC  
Gly Pro Gly Ile Glu Glu Gly Trp Leu Pro Pro Leu Glu Ala Ile Glu Arg Ile

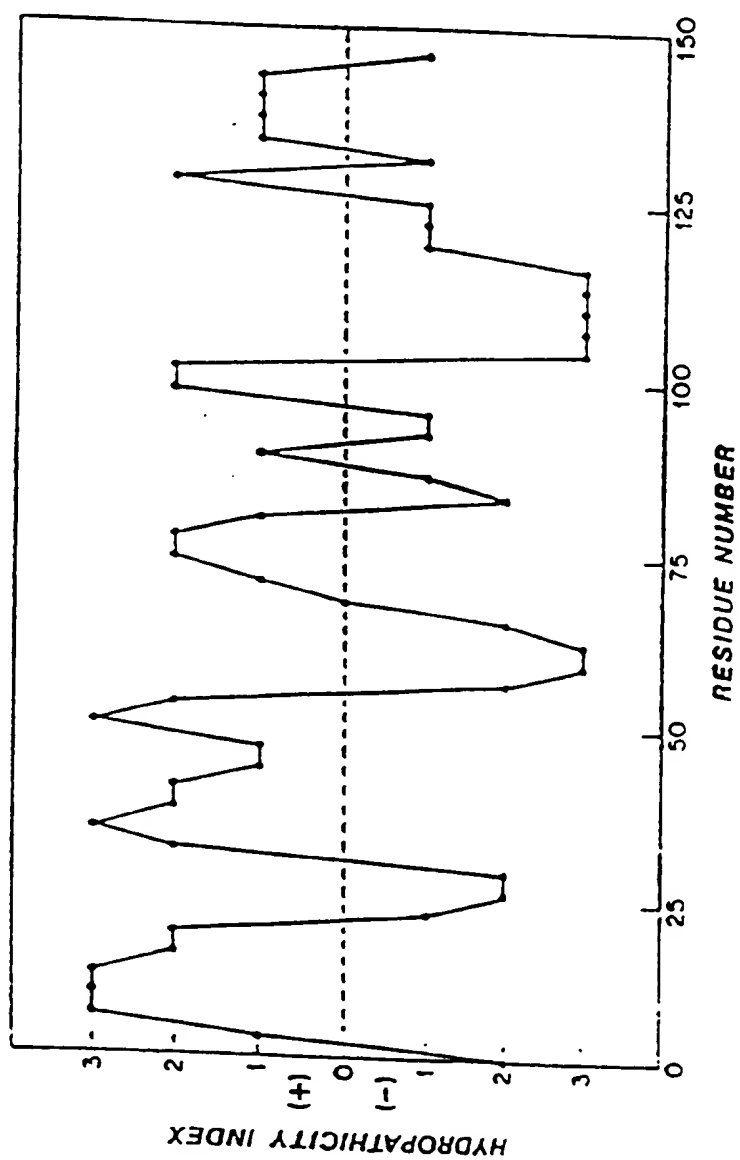
1500 1510 1520 1530  
GAG CTC ATC CGT CGC CGC ATG TCT ACC CTG TAC CGC TCG CAT CGC GAC ...  
Glu Val Ile Arg Gly Pro Met Ser Thr Leu Tyr Gly Ser Asp Ala Asp

irgA

CONSENSUS

GAAATTAAGAATAATTATC  
|| | | | ||| |||||  
GATAATGATAATCATTATC

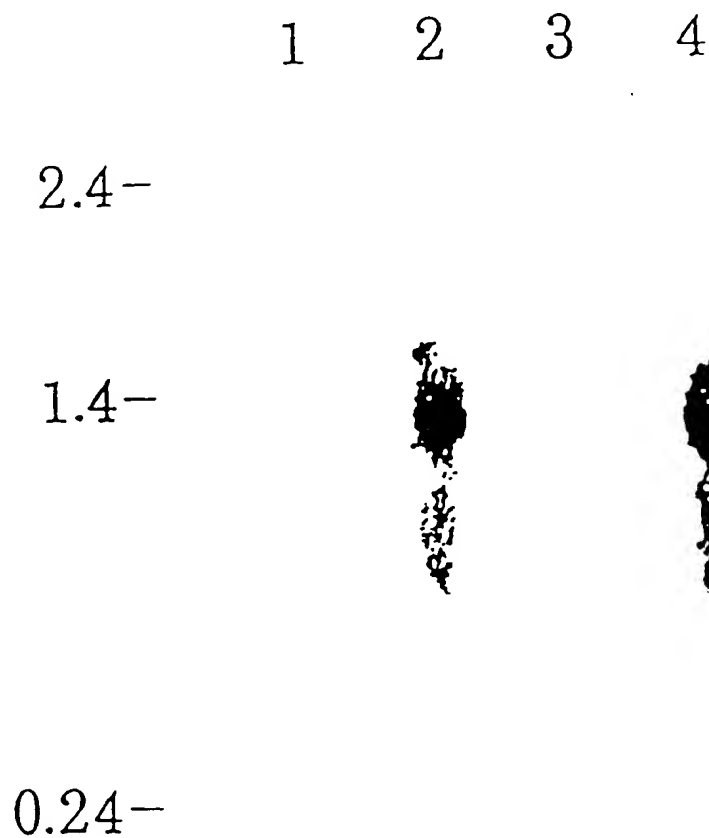
FIG. 10 10/15





IrgA	132	Q A I E R I E V I R G P M S
FepA	139	E M I E R I E V I R G P A R

12/15



10 20 30 40 50 \* 60 70 80  
GGCGATGATGATTCACATTTACCTCTTAAATGATAATTTCTTAATTTGACAGGGGATTTTCATATA  
-35 -10

90 100 110 120 130 140 150 160  
TTCTTACTGGAGATAGATGACACATGCAATGTTTGGTCCAAATATGGAAGCTCTGTGTAATTTCTTTAGGCTC  
SD

170 Bell 190 HindIII 200 210 220  
AAATACCT ATG CAA GAT CTC AGC GGC GTA AAA GCT TTC CAT GGC CTT TCC CAA CAT AAG AGC  
Met Gln Asp Leu Ser Ala Val Lys Ala Phe His Ala Leu Cys Gln His Lys Ser

230 240 250 260 270 280  
CTG ACT GGC GCT GGC AAA GGC CTT GAA CAG OCT AAA TCC ACC CTG AGT GGC CGT TTG GGC  
Leu Thr Ala Ala Ala Lys Ala Leu Glu Gln Pro Lys Ser Thr Leu Ser Arg Arg Leu Ala

290 300 HindIII 320 330 340  
CAA CTT GAA GAG GAC TTG GGA CAA AGC TTG TTG ATG CGT CAA GGC AAC GGC TTA ACC CTC  
Gln Leu Glu Glu Asp Leu Gly Gln Ser Leu Leu Met Arg Gln Gly Asn Arg Leu Thr Leu

350 360 370 380 390 Bell 400  
ACC AAA GCA GGA GAA GTG TTT GCG GTT TAC TCG CAG CAA CTA CTT GAA CTG GGC AAT AAA  
Thr Lys Ala Gly Glu Val Phe Ala Val Tyr Ser Glu Gln Leu Leu Glu Leu Ala Asn Lys

410 420 430 440 450 460  
AGT CAG GAA GCG TTG CAA GAA TTG AAC AAT CAA GTG ACT GGC GAA CTC ACC CTG CTG GTA  
Ser Gln Glu Ala Leu Gln Glu Leu Asn Asn Gln Val Thr Gly Glu Leu Thr Val Val

470 480 490 500 510 520  
CAC CCC AAT TTG ATC GGC GGC TCG CTC AGC CAA GTA TTG GAT GAG TTT ATG CAG CAG CAT  
His Pro Asn Leu Ile Arg Gly Trp Leu Ser Gln Val Leu Asp Glu Phe Met Gln Gln His

530 AccI 550 560 570 580  
TCG ACA TTG AAG ATC GGT CTA CTC AGC CAG TTT CAA CAC AGT GAT GAG CTG TTT GAG GGC  
Ser Thr Leu Lys Ile Arg Leu Leu Ser Gln Phe Gln His Ser Asp Glu Val Phe Glu Pro

590 600 610 NcoI 630  
GAT TTG ATC ATT TCG ATT GAA CAC GGC GGC CCC ATG GGT TAT GGC AAA GAA GGC TTA GGC  
Asp Leu Ile Ile Trp Ile Glu His Ala Ala Pro Met Gly Tyr Arg Lys Glu Arg Leu Gly

650 660 670 SspI 690 700  
TAT TCG GGC TAT GGC ACT TAC GGC TCT CCC AAA TAT TTG GGC CAT CCA GAT AAA GGC ACC  
Tyr Trp Arg Tyr Ala Thr Tyr Ala Ser Pro Lys Tyr Leu Ala His Arg Asp Lys Pro Thr

710 720 NcoI 740 750 760  
CAT OCT GGT GAG CTG ATT CAT CAC CCA TCG ATT GAT TTT ATT GCT TGT GGC GGC GGC GAG  
His Pro Arg Glu Leu Ile His His Pro Trp Ile Asp Phe Ile Ala Cys Arg Arg Ala Glu

770 780 790 800 810 820  
CTT GAA CTG CAC CAT CCA GAG TTC GGC AGT TAT TCA CTG CCA CCA CTA GAG AGT GGT TTA  
Leu Glu Leu His His Pro Glu Phe Gly Ser Tyr Ser Leu Pro Ala Leu Glu Ser Arg Leu

830 840 850 860 870 880  
CAA AGC GAT AAT CTT GGC ATG CAA GGC GAT GGC ATT GCT AAA GGT GGT GGT ATT GGT TTG  
Gln Ser Asp Asn Leu Ala Met Gln Ala Asp Ala Ile Ala Lys Gly Arg Gly Ile Gly Leu

890 900 910 920 930 940  
CTT CCC ACT TCG TTT GGC AAT GGT TTT GAA AGC GGC CAT GGC GGC AGC CTC ATT OCT TCG  
Leu Pro Thr Trp Phe Ala Asn Gly Phe Glu Thr Ala His Pro Gly Ser Leu Ile Pro Cys

HincII 950 960 970 980 990 1000  
CTC AAC CCA TCG CAA TCA CAG CCA ACA GAA ATC AAC TCC TTC TAT GGC CTC GGT GGT CAC  
Val Asn Gly Trp Gln Ser Gln Pro Thr Glu Ile Asn Cys Phe Tyr Pro Leu Gly Arg His

1010 1020 1030 1040 1050 1060  
CCA CTT GGC CTG GGC CTA TTT ATT GAT GGC CTC GGC CAA GCA AGC GGC GAT GAG TCG CAA  
Pro Leu Arg Leu Arg Leu Phe Ile Asp Ala Leu Arg Gln Ala Arg Pro Asp Glu Trp Gln

1070 1080 1090 GlaI  
TAA AAAATCCCCCGGACGGGATTTTTCATCGAT

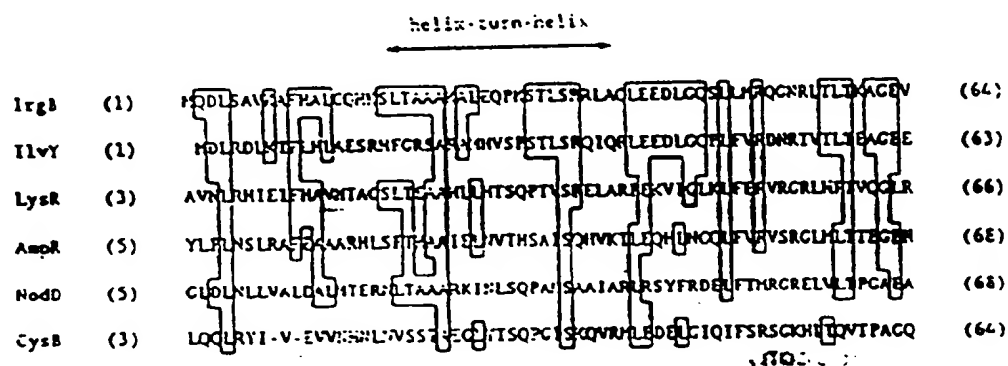
FIG. 14 14/15

5' CCG CGA TCG ATT GAA TCT GGA CAT TTACCACTCCTTTAAAT CATAATTATTCTTAAATTG  
 3' CCC CCT ACC TAA CTT AGA CCT GTA AATGGTCAGGAAATTTA CTATTAATAAGAATTAAAGT  
 Pro Ser Pro Asn Phe Arg Ser Met SD \*  
 ← IrgA

GAAGCGGATTATTCATATATTCTGTACTGGAGATAGATCGACACATGGAATAGTTCCGGTCCAAATATGG  
 CTTCGCCTAATAAGTATATAAGACATGACCTCTATCTAGCTGTGTACCTTATCAAGCCAGGTTTATACC  
 -10

AAGCTCTGTGGTAATTATTCTTTAAGGGTCAAATACCT ATG CAA GAT CTC AGC GCC GTA 3'  
 TTCCAGACACCATTAAATAAGAAATTCACAGTTTATGGA TAC GTT CTA GAG TCG CGG CAT 5'  
 SD IrgB → Met Gln Asp Leu Ser Ala Val

FIG. 15 15/15



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09592

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): C12N 1/20, 1/00; A61K 39/00		
US CL : 435/243, 252.3; 424/88, 92		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	U.S. CL. 435/ 243, 252.3; 424/88, 92	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
Dialog files: 5, 155		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
X	Infection and Immunity, Vol 58, No. 1, issued January 1990, Goldberg et al, "Identification of an Ironn-Regulated...Mutagenesis", pages 55-60, see entire document.	1-5,9-13,17-19
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:<sup>16</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>		Date of Mailing of this International Search Report <sup>2</sup>
18 MARCH 1992		30 MAR 1992
International Searching Authority <sup>1</sup>		Signature of Authorized Officer <sup>20</sup>
ISA/US		H. F. Sidberry <i>[Signature]</i>